Morphological Correlates of Long-term Potentiation and Ageing in the Hippocampus of Rats

Thesis

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Morphological Correlates of Long-term Potentiation and Ageing in the Hippocampus of Rats

A thesis submitted in partial fulfillment of The Requirements for The Degree of

Doctor of Philosophy in

The Brain and Behaviour Research Group

By

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15th- February -1999'.

DATE OF SUBMISSION : 23 FEBRUARY 1999

DATE OF AWARD : 23 SEPTEMBER 1999

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This thesis is dedicated to my Bhagawan, Parents, Sister, Sister-in-law, Brothers & my Wife.

Acknowledgement

I would like to thank my supervisor Prof. Mike Stewart for his friendship, support, for sensitive guidance through my studies and his invaluable help concerning the presentation of this thesis

I am grateful to Dr. Dimitri Rusakov for his very useful ideas particularly for his expertise in statistics and theoretical aspects of morphology.

I also thank especially the head of the electron microscopy unit, Mrs. Heather Davies, for providing training in electron microscopy and light microscopy and her always friendly support and patience.

I wish to thank all the people who have helped to make this work possible, particularly Dr. Marina Lynch, A. Kelly (Department of Physiology, Trinity College Dublin, Dublin; Ireland), Prof. Steven Rose, Elaine Harrison, Jacki Brown, Steve Walters, Dr. Rachel Bourne, Karine Cambon, Mark Bressler, Dipesh Chaudhury, Lindsay Roberts and Claire Kendal. I apologize to everyone I have missed out.

I am really grateful to my parents, brothers, sister and sister-in-law for their constant support. Finally, I acknowledge with gratitude, the receipt of an Open University Postgraduate Studentship during my studies.

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General Discussion

List of Abbreviation

Anatomical

DG	Dentate gyrus
Sch	Schaffer-collateral
comm	Commisural fibers
mf	Mossy fibers
fim	Fimbria
pp	Perforant pathway
EC	Entorhinal cortex
CA	Cornu ammonis
NCAM	Neural cell adhesion molecule
NF	Neurofilament
GFAP	Glial Fibrillary Acidic Protein.
HP	hippocampus
MAP2	Microtubule associated proteins
CaMKII$\propto$	Ca^{2+}/ Calmodulin-dependent kinase II
Arc	Activity Regulated Cytoskeletal Protein
CPE	Cytoplasmic Polyadenlation Element
CPEB	Cytoplasmic Polyadenylation Element Binding Protein

General

AMPA	γ-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	Analysis of variance
CNS	Central nervous system
c-fos	Cellular fos mRNA
EPSP	electro-shock
GABA	γ-aminobutyric acid
HRP	Horseradish peroxidase
i.c.	Intracranial injection
i.p.	Intraperitoneal
c-jun	Cellular jun mRNA

LCN	Local circuit neurone
LTM	Long-term memory
LTP	Long-term potentiation
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
N_{syn}	Synaptic number
Nv_{syn}	Synaptic density per μm^3
PBS	Phosphate buffer saline
PKA	Protein kinase A
PKC	Protein Kinase C
PSD	Postsynaptic density
SEM	Standard error of the mean
Sh	Shaft synapses
Sp	Spine synapses
STM	Short term memory
PS	Perforated synapses
SCZ	Synaptic active zone
SA	Spine apparatus

Publications Arising from the Work

Abstracts:

1. Dhanrajan T. M., Lynch M. A., Kelly A., Stewart M.G. and Rusakov D.A. Alterations in dendritic spine morphology in the hippocampus of aged rats 45 minutes after induction of LTP. Brain Res. Assoc. Abstr No: 29.05; Vol. 14: p67,1997.
2. Stewart M.G., Dhanrajan T. M., Lynch M. A., Kelly A and Rusakov D.A. Plasticity of dendritic spines in the hippocampus of aged rats 45 minutes after LTP induction. Society for Neurscience. Abstr No: 263.12; Vol. 23: 2 1997.
3. Dhanrajan T. M., Stewart M.G., Lynch M. A., Kelly A. and Rusakov D.A. Plasticity of dendritic spines in the hippocampus of young and aged rats 45 minutes after LTP induction. Forum of European Neuroscience, European Journal of Neuroscience Vol.:10 Supplement 10 Abst. No:170.02; p391,1998.
4. Dhanrajan T. M., Stewart M.G., Lynch M. A., Kelly A. and Rusakov D.A. Correlation between dendritic spine morphology, synaptic density, and the ability of aged rats to sustain LTP in dentate gyrus, 45 min after tetanic stimulation of the perforant path. The 6th Neuropharmacology Conference, Abstract No: 4.21; 1998
5. Dhanrajan T. M., Stewart M. G., Lynch M. A., and Kelly A. Correlation between dendritic spine morphology and the ability of aged rats to sustain LTP in the dentate gyrus. Society for Neuroscience. Abstr No: 132.7; Vol. 24: 1 1997.

Abstract

This thesis has examined age-dependent changes in neural plasticity in rat hippocampal dentate gyrus using unbiased morphological techniques at light and electron microscope level. It considers whether there is a morphological basis to explain why some aged animals sustain, whilst others fail to sustain, potentiation in the dentate gyrus of the hippocampus after unilateral induction of LTP, 45 minutes after stimulation of the perforant pathway. Previous data in young adult rats (5 months old) have demonstrated that the morphology of dendritic spines and synapses within the hippocampus is altered at 10-30 minutes, and 24 hrs following LTP induction. The data obtained in the present study suggest that differences in spine and synaptic morphological parameters appear to be correlated with the ability to maintain LTP in the aged rats. Those maintaining LTP had a tendency to longer spine length, and a decrease in spine and synaptic densities, there was a significant increase in the number of complex axospinous perforated synapses.

Here, in young rats, LTP resulted in a significant increase in the density of spine synapses and total synaptic density. The mean spine density was also higher in the stimulated hemisphere, but spine length decreased. However, there was a significant increase in the number of bifurcating spines and axospinous perforated complex synapses in the stimulated compared to the contralateral control hemisphere.

An age-dependent comparison indicated that spine density and synaptic densities are significantly higher in the younger rats, but spine length was significantly greater in

the aged rats. LTP does not seem to cause morphological changes *per se* at the time examined post potentiation. However, an important finding of the present study is that the percentage of axospinous complex perforated synapses is significantly higher in the stimulated hemisphere of aged and young rats that sustained LTP compared to those that did not. The percentage of branched spines and simple axospinous perforated synapses is significantly higher in both the stimulated & unstimulated hemisphere of aged rats that failed to sustain LTP. Therefore a proportion of the branched spines and perforated synapses would appear to be the result of high frequency stimulation, rather than LTP induction *per se*.

Chapter 1

Introduction

"They live by memory rather than by hope; for what is left to them of life is but little as compared with the long past; and hope is of the future, memory of the past. This, again is the cause of their verbosity; they are continually talking of the past, because they enjoy remembering it."

Rhetorica II.1390a.10 (from the English translations of Ross, 1928).

1.1 Anatomy of Hippocampus:

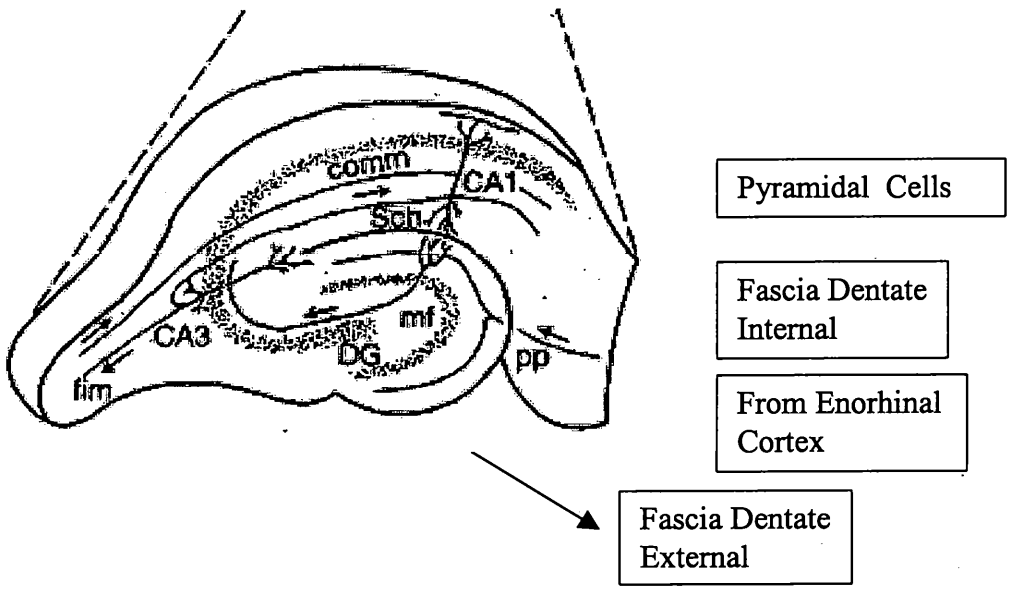
The hippocampus has been the central point of memory studies in the mammalian brain, since Milner and Penfield's landmark 1956 study (Milner and Penfield, 1956). On the basis of clinical observations of brain-damaged individuals, and electrical stimulation during brain surgery, temporal lobe structures were implicated in human memory (Penfield and Milner, 1958). Subsequent studies in animals have further implicated the hippocampus in learning and memory (Squire, 1992; Eichenbaum, *et al.*, 1986). As discussed below, much of the interest in the hippocampus comes from an appreciation of its anatomical circuitry. On the phylogenetic level, the hippocampus only appears distinctly in the mammalian brain, although hippocampal homologies can be found in the brains of birds, reptiles, and amphibians (Angevine, 1975).

The importance of the hippocampus in recognition memory formation stems from humans and animal models developed to mimic human amnesia. Recent data in monkeys indicates that in a delayed non-matching to sample tasks, hippocampal lesions produced significant impairment in recognition memory (Zola-Morgan and Squire, 1986). Also clinical cases reported anterograde amnesia for verbal and non-verbal materials following bilateral lesions of the CA1 subfield of the hippocampus (Zola-Morgan and Squire, 1986; Squire, 1992). The size and position of the hippocampus highlights that it plays potentially an important role in total brain function (Squire, 1992).

The geometry of the hippocampus is unique in the mammalian CNS. The hippocampus is bilaterally symmetrical structure shaped like cashew. It is composed of two interdigitating fields: the hippocampus proper (*cornu ammonis*) and the dentate gyrus (*fascia dentate*) (Fig1.1). Both fields are folded into a shape reminiscent of the letter C.

The hippocampus proper is often associated with the so-called CA fields. This layer is formed by a large flap of tightly packed pyramidal cells that is folded and tucked under the edges of the neocortex.

Fig1.1: A diagrammatic representation of the hippocampus. It shows the pyramidal cells in the CA1 and CA3 fields and granule cells in the dentate gyrus (DG).



The second sheet of neurons in the hippocampal formation is the dentate gyrus, which contains basket shaped, tightly packed neurons called granule cells. The fascia dentate has an internal or buried blade and an external or exposed blade. The sheets of pyramidal neurons are not continuous with the dentate gyrus. The dentate gyrus bends around the end of the pyramidal sheet resembling the top of a tooth in cross section, hence it derives its name (Amaral, *et al.* 1990).

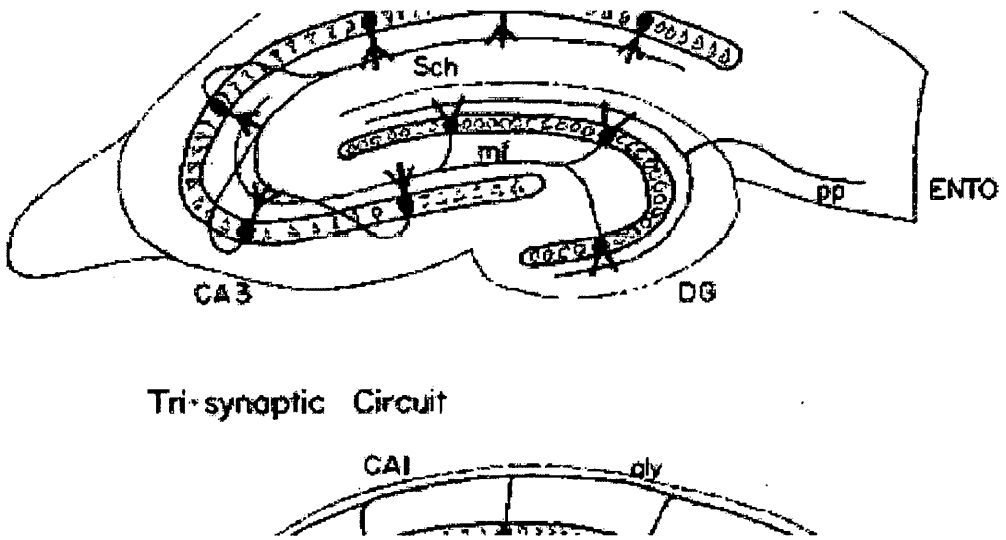
One striking feature of hippocampal circuitry is the pattern of afferent termination. Major hippocampal afferents originating from the entorhinal cortex and contralateral and ipsilateral hippocampal subfields synapse on the dendrites of granule and pyramidal cells respectively in a laminated pattern (Raisman, *et al.*, 1965; Amaral, 1991). The dentate

gyrus exemplifies this pattern of laminated afferents inputs. Hippocampal commissural and associational fibers synapse within the proximal one-third of the granule cell dendritic field (the dendrites closest to the cell body layer) (Zimmer, 1971). The massive entorhinal cortex projections from the perforant path (Figure 1.1) terminate topographically in the outer two-thirds of the dendritic field; the lateral entorhinal cortex projects most distally, the medial entorhinal cortex most proximally, and the intermediate entorhinal area to a position between the medial and lateral entorhinal termination zones (Steward, 1976). Afferent lamination is also an organizing principal in the hippocampal proper.

Another important feature of the hippocampus is the intrinsic circuitry, or the manner in which units within and between the sheets of cells are connected (Amaral and Witter, 1989). Physiological studies identified a circuit contained within the sheets of cells, oriented transverse to the longitudinal (or septo-temporal) axis of the hippocampus (Andersen, *et al.*, 1971). This is known as the **trisynaptic circuit** (Figure 1.2) -strips of cells that form a functional circuit and possess a lamellar organization (Andersen, *et al.*, 1971a). (i) The *perforant pathway* runs from the subiculum to the granule cells in the hilus of the dentate gyrus. (ii) The axons of the granule cells form a bundle, the *mossy fiber pathway* that runs to the pyramidal cells lying in the CA3 region of the hippocampus. (iii) the pyramidal cells in the CA3 region send excitatory collaterals, the *Schaffer collaterals*, to the pyramidal cells in CA1. The hippocampal slice preparation takes advantage of the trisynaptic anatomy of the hippocampus, preserving this circuit in

a 300-400-μm- thick slice of brain tissue that can be maintained, manipulated and studied for hours under closely controlled conditions.

Figure 1.2 Diagrammatic representation of the trisynaptic circuit of the hippocampus. It also shows the cornu ammonis (CA fields) and dentate gyrus (DG). alv, alveus; mf, mossy fibers; pp, perforant path; Sch, Schaffer collaterals and entorhinal cortex.



1.2 Effects of Selective Hippocampal Lesion Studies:

Cognitive studies on memory impaired patients provided valuable information about the organization of memory function (Baddeley, 1982; Cermak, 1982; Milner, 1972; Schacter, 1985; Squire, 1986; Weiskrantz, 1987). In particular, work with the noted amnesic patient H.M.(Scoville & Milner, 1957) demonstrated that memory depends on the integrity of the medial temporal lobe. Temporal lesions to humans and monkeys, especially to the hippocampus, appear to result in a loss of memory for events intervening between the time of surgery and the present (Alvarez-Royo *et al.*, 1992; Sass *et al.*, 1992). Memories of the remote past seem unaffected, but formation of new memories are

lost or impaired from the time of surgery. Recent studies have shown that global amnesia results from limited damage within the hippocampus itself (Zola-Morgan *et al.*, 1986) or including the hippocampus and dentate gyrus and saving most or all of the adjoining cortex and other medial temporal structures (Vargha-Khadem *et al.*, 1997). These conclusions suggest that the hippocampus plays an important role in memory formation for a broad domain of information in humans. Various theories that have sought to understand 'the role of hippocampus' in learning and memory are discussed below.

Numerous studies have sought to interpret the role of hippocampal information processing, using electrophysiological, neuropsychological and behavioral model approaches in animals. Some of these earliest animal studies performed lesions restricted to different regions of the hippocampus to study functional differences (Isaacson, 1972; Alvarez-Royo, *et al.*1992). Selective aspiration damage to hippocampal cell fields and projections were studied by Jarrard (1978a). These experiments involved rats with damage to the CA1 field, to the overlying alveus, to the alveus alone, and to the fimbria (interrupting most of the extra-hippocampal projections from the CA3-CA4 cell field), as well as rats with complete aspirational lesions of the hippocampus (Gray *et al.*1977; Jarrard *et al.*1978a, b). A majority of the tasks adopted had earlier been shown to be unstable following large hippocampal damage, and it was postulated that by applying specific regional lesion in a much more selective, this would enable to assess the magnitude of behaviour to be correlated with hippocampal subfields (Eichebaum, *et al.*1986; Dudai, 1994).

Animals with lesions transecting the fimbria were shown to be affected in a way resembling those with broad hippocampal lesions; both groups were more alert than

others at night and daylight, and both were affected by food deprivation (Jarrard, 1980). Moreover, animals with lesions transecting the fimbria or the complete hippocampus were facilitated in their acquisition of shuttle-box avoidance, and were also impaired in a differential reinforcement task, by a low rate of responding (Jarrard, 1980; Freeman and Stanton, 1991). Extensive damage to the hippocampus, and selective damage to the CA1 pyramidal cell field and the alveus, resulted in impaired spatial reversal learning. In contrast to its involvement in long term memory formation loss of recent memory is not an observable characteristic of hippocampal damage (Jarrad, *et al.*, 1984).

The inability to detect recent memory impairments in other vertebrate species (i.e. chicks) has constantly been a theoretical hurdle that has led researchers to consider the fundamental differences in hippocampal functions between man and other animals (Squire, 1992).

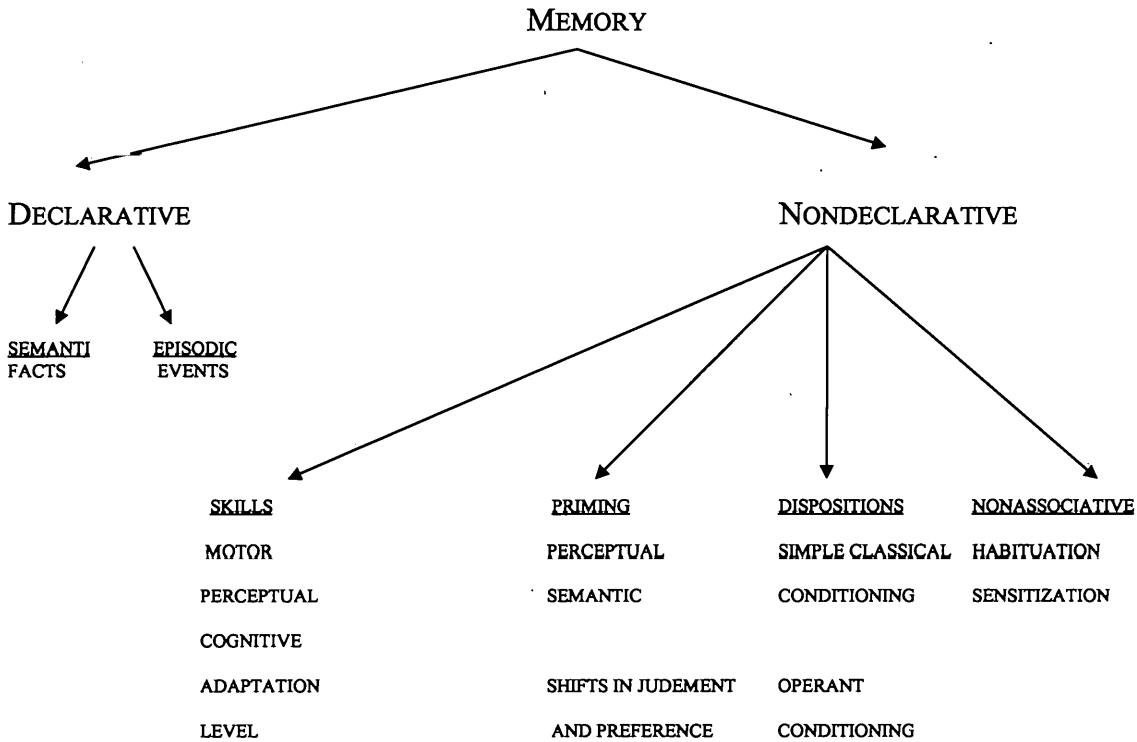
1.3 Learning and Memory:

Learning is the process of acquiring information of the world and memory is defined as the retention and storage of knowledge procured through learning. There are two types of learning paradigms: Associative and Non-associative learning. The establishment of a relationship between two events involves associative learning. The more basic learning processes, involves a response possibility due to the effects of a single event is known as non-associative learning. Associative learning is generally subdivided into classical versus instrumental conditioning or learning. The examples of non-associative learning are habituation, dishabituation and sensitization.

Identifying the role and nature of memory processing by the hippocampus has proved a fierce venture. The basic idea is that memory is not a single entity but consists of

numerous separate entities that depend on different brain systems (Squire and Zola-Morgan, 1996). Substantial progress has been made in interpreting memory storage mechanisms in invertebrate systems (Abrams *et al.*1991); however related progress has yet to be made in studies with the mammalian brain. As emphasized many years ago (Lasley, 1929), the primary difficulty in examination of memory mechanisms in the mammalian brain is localization of memory storage. Mechanisms of memory storage cannot be investigated until the memory storage sites, whether localized or distributed, have been localised. Memories may involve ensembles of neurons, even if this is within a defined region of the brain. Memory can be divided into several types (Squire, 1986,1987) (Fig1.3). The kind of memory that depends specifically on the hippocampus and related structures has been termed *declarative memory* is the sense that one can bring to mind or declare the content of this kind of memory (Anderson, 1976). The term *procedural memory* was traditionally used to contrast with declarative memory (Winograd, 1975), and describes the knowledge acquired during skill learning, but it is not clear that this same term is useful for the many examples of learning and memory now know to be independent of the hippocampus. Thus the term *nondeclarative memory* was introduced to collectively describe these memory abilites (Squire and Zola-Morgan, 1988). As discussed above the fact lies in the distinction between the capacity for conscious recollection of facts and events (declarative memory) and heterogeneous collection of nonconsious learning capacities (non-declarative memory) that is expressed through performance. The studies conducted on amnesic patients are the best examples that distinguishing between the types of memory (Squire and Zola-Morgan, 1996).

Figure 1.3: A schematic view of memory taxonomy (from Zola-Morgan and Squire, 1990).



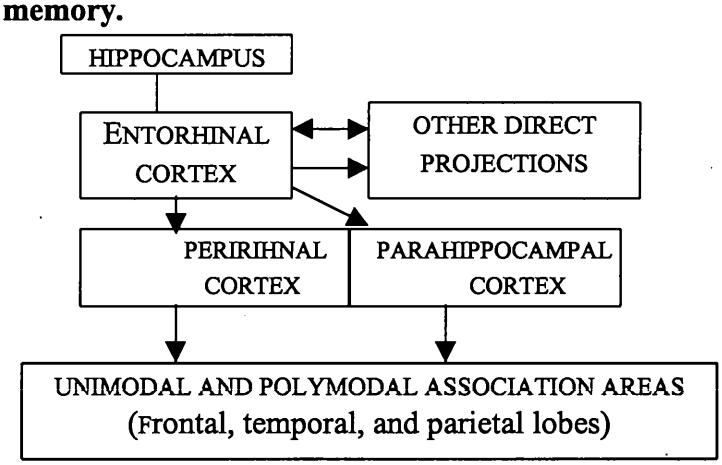
The structures in the medial temporal lobe that essential for declarative memory has largely come from the work carried out on monkeys and rats. The cortial regions adjacent to the hippocampal formation, including entorhinal, perirhinal, and parahippocampal cortices, are essential for medial temporal lobe memory system (Suquire and Zola-Morgan, 1996). The hippocampus is essential for relational, contextual, spatial, as well as olfactory learning in lower mammals (Eichenbaum, *et al.*, 1986; Lynch, 1986; O'Keefe and Nadel, 1978 and Squire, 1992) (Fig 1.4). The cerebral cortex is believed to be the intended reservoir of long-term declarative memories although definitive proof endorsing this view is absent. The amygdala plays an important function in introductory learning of conditioned heart momentum and blood pressure, conditioned potentiation of surprise, conditioned frigid response to sound, and instrumental avoidance (Faneselow, *et al.*,

1991). The above anatomical regions by virtue of their widespread and reciprocal connections with neocortex, could be important for establishing long-term memory for facts and events (declarative memory). The medial tempoal could be a bridge binding the storage regions in the neocortex that could represent a whole memory (Squire and Zola-Morgan, 1991). Hence, it could be a temporary place of long-term storage (McGaugh, 1989; LeDoux, 1987; Rogan and LeDoux, 1995). Fresh information indicates that the basal ganglia could play a significant part in certain aspects of instrumental learning (Packard, *et al.*, 1989). A section of the cerebellum is essential for primary delay classical conditioning of distinct motor responses. Conversely, it is conceivable that together these memory systems compliment various aspects of learning, besides individual systems playing their distinct part (Lavond, *et al.*, 1993). But once the centre, or loci, of a given type of long-term memory (i.e. Declarative or Procedural) has been established, it will be feasible to study in detail the mechanisms of memory storage.

The focus of all this discussion on normal learning and memory processes probable needs to be considered with reference to their own developmental time course (not to include possible performance confounds) and that alterations in memory capacity with age can be informational in their own right, highlighting the characteristics crucial for information-processing system. Ageing is a common process to all organisms but in spite of its completeness, understanding of the cellular and molecular alteration which accompy ageing is poor. In reference to the brain, ageing has four interconnected theories that have received notable attention; these are (1) the free radical hypothesis, (2) the membrane hypothesis, (3) Calcium hypothesis and (4) the glucocorticoid theory. During normal ageing of the brain its weight and volume decrease significantly and the ventricles of the

brain enlarges (Lynch, 1998). These could include chemical/ structural alteration at dendritic spines, synapses and probably long-lasting changes in gene expression in the appropriate neurons. Loss of neurons from the ageing human brain is also accompanied by reduction in biochemical markers (Bowen and Davison, 1980). The possible relationship between age-related behvaioral changes and neurochemical systems involves the study of the neurotransmitter acetylcholine (Bartus *et al*, 1982). Although multiple neuro-transmitter systems are clearly involved both in normal aging and in Alzheimer'disease (Chan-Palay, 1988). Determining these mechanisms, nevertheless, will not tell us what the memory actually is *per se*; although the composition of a neuronal memory store may be resolved to a greater extent by a precise characterization of the neural networks that subserve the memory. Hence, attention is focussed on analysis of the morphological changes that might underlie age-related alterations in hippocampal function, particularly induction of long-term potentiation (LTP).

Figure 1.4: A schematic view of the structures of the medial temporal lobe important for declarative memory.



1.4 Introduction – Experimental Models:

The need for rodent behavioral tests that could sample simple forms of cognition-linked learning and also respond appropriately to lesions in the hippocampus, amygdala, and dorsomedial nucleus of the thalamus was illustrated by the discovery that different neural systems in humans and primates mediate different aspects of memory (Cotman and Lynch, 1989). Animal models of amnesia have offered numerous insights into the neural substrates of different types of memories. The loss of explicit declarative and episodic memories following hippocampal-region damage has been the focus of analyses of amnesia, significant insights into amnesia can also be gained by examining hippocampal function in simple procedural, or habit–based, associative learning tasks (Vnek and Rothblat, 1996). It is known that simple forms of associative learning tasks are not impaired by hippocampal damage, but complex tasks that necessitate sensitivity to unreinforced stimuli, configurations of reoccurring stimuli, or contextual information are impaired by hippocampal damage (Gluck, *et al.*, 1997). Animal models of normal aging have been used to study the neurobiological structures and connections involved in memory functions, i.e. hippocampal/cortical systems. As a model, spatial learning is dependent upon the completeness of the hippocampus, a region that is damaged in humans and Alzheimer's disease (AD) (Squire, 1986). Spatial learning tasks, such as the morris water maze, radial arm maze and T-maze, have been used to screen aged and young rats for cognitive status prior to extracting basic principles from these models in which molecular and anatomical details can be studied (Cotman and Lynch, 1989).

1.4.1 Spatial Learning:

A unique approach in animal models of memory and ageing is to select a behaviour that has relevance to human behaviour (Barnes, 1980). Provided this behaviour alters in an age-dependent fashion in both the animal model and the human case, the possibility of producing assumptions from the nonhuman model is facilitated, particularly when proof exists that similar brain and behaviour relationship exits between two species (Olton, 1976; Squire, 1988). Spatial memory impairments appear in both elderly humans and in old rats, and there is evidence that age-related alterations in the hippocampal formation could be liable for the detected behavioral changes in both species (Bruce and Herman, 1983; Walsh, *et al.*, 1981).

The type of learning and memory that will be referred to as "spatial" involves the capability to accomplish and to preserve associations of environmental features. These associations permit an organism to manoeuvre efficiently around space. The initial suggestions that elderly humans might encounter difficulty with remembering spatial environmental relationships were anecdotal. However, this has been studied more recently in experiments, by testing "healthy" older adults. It was established that older human beings do not retain spatial representations of their environments as well as young adults (Zelinski and Light, 1988).

The earliest animal experiments on spatial memory concerned a series of experiments on rats of different ages were those of Stone, (1929a,b) who concluded that, as a whole, older animals did not indicate obvious cognitive impairments compared with his younger animals. A closer examination of the specific data of the rats, nevertheless, suggested that a small portion of his oldest animals did seem to reveal few deficits. However, Stone's

rats were a somewhat long-lived strain (3-yr average life span), and were tested at 2- yr of age, so this data most likely reflects the behavior of rats around late adulthood but not old age. Recent studies using multiple-unit T-mazes *and* shorter -lived rat strains consistently found age-related deficits on learning this task (Goldman, *et al.*, 1987). Chronological age is still apparently the best overall predictor of the functional or biological age of the animal (Costa and McCrae, 1980). For humans old age is almost regularly considered to be above 65yr, and for rats past the point of 50% mortality for the strain in question, that is about 2yr for the rats most frequently used in laboratories today.

1.5 Morris Water Maze:

The Morris water task has likewise been utilized to test spatial memory of young and old rats and in numerous ways is identical to the circular platform maze. In this task the animal must locate an unseen escape platform in a massive water filled pool, in which cues outside the pool aid navigation to the platform position (Morris, 1981). Aged animals constantly exhibit poorer memory of the concealed or secret platform site than do younger animals (Rapp, *et al.*, 1987; Linder and Schallert, 1988). Provided however, the escape platform is lifted over the surface of the water so that the task is momentarily nonspatial, then the old rats' ability is similar to that of the younger rats (Rapp, *et al.*, 1987). Taken in connection with the different spatial tasks considered earlier, spatial information handling appears to pose specific problems for older rats, as for older humans.

1.6 Radial Arm Maze:

Various other tasks have been adopted to evaluate the spatial memory of rats of different ages. In the radial maze designed by Olton and Samuelson (1976), eight arms radiate

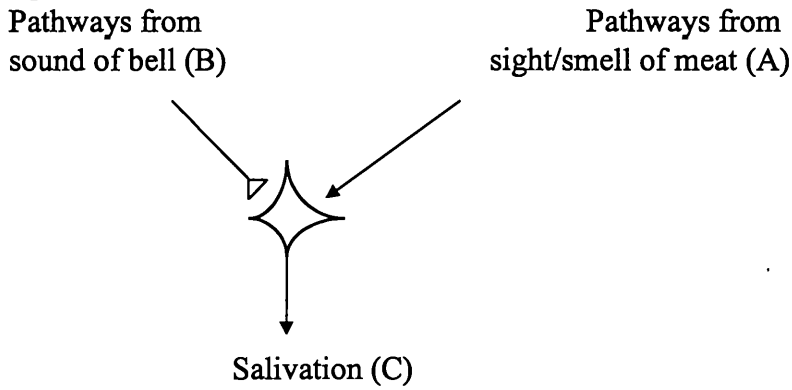
from a central platform, the end of each having a reward. The problem posed by this task is to acquire a reward at every arm end without re-entering any of the arms in which the rewards have been acquired earlier. Hence, the animal needs to recall which spatial position it has visited lately and then proceed to places not yet visited on that specific path. The recollection of where they have been, and where to go next using visual signs exterior to the maze, has been termed spatial working memory. Possibly one of the best-replicated reports in the animal memory literature in gerontology is that aged rats require many more trials to attain a given "criterion" or mastery of performance on the radial maze than do young animals (Barnes, *et al.*1980; Geinisman, *et al.*1986a, b). Thus indicating that they have a problem in learning the above task (Klein and Michel, 1977).

1.7 Hebbs Theory:

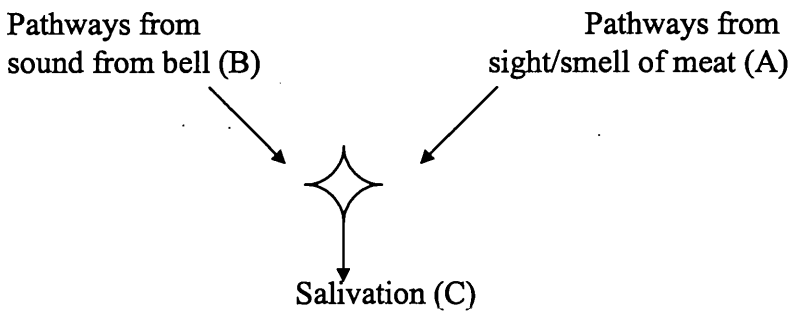
The ultimate way in which memories are encoded is likely to involve a change in synaptic efficacy. How this might occur is still based on ideas from Donald Hebb (Hebb, 1949). The properties of any learning system can be inferred from the input-output relationships, in other words (stimulus and resulting behavioral response), treating every neuronal mechanism as a black box (Dudai 1989). Hebb (1949) presumed that synaptic function is strengthened by co-activation of a presynaptic fibre and its postsynaptic neurone. For example, the synaptic transmission from a presynaptic fibre, a, to its postsynaptic neurone, b, increases in efficiency when excitation occurs concurrently and repeatedly in both elements. This hypothesis of learning process where simultaneously activation of pre- and postsynaptic elements occurs is termed the Hebbian synapse. As illustrated in Fig 1.5

Figure 1.5: Schematic drawing of Hebb's proposed mechanism for altered synaptic efficacy leading to memory formation. (D. O. Hebb: The organisation of behaviour, Wiley, 1949; pp62-63).

Before Learning:



After Learning: (Strengthening of Synaptic Circuitry between B and C neurons).



In the above figure 1.5, suppose C is a neuron responsible for salivation, A is a pathway from the olfactory system that activates C on the smell of food, and B is a weak pathway from the auditory system which responds to the sound of a bell, then a mechanical action would result in the animal now responding to the sound of the bell by salivating. Hence, based on Hebbian theory strengthening of connections could be possible if some growth or modification in the synaptic bouton B and C occurred.

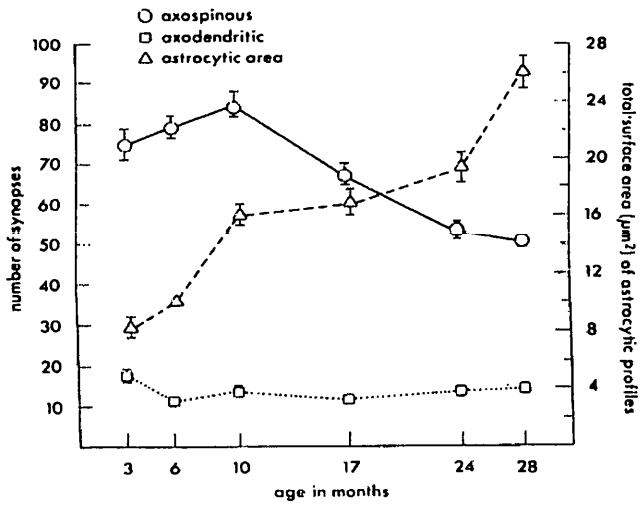
1.8 Ageing:

The distribution and density of dendritic spines and synapses is of direct functional significance in ageing (Barnes, 1980; Geinisman, 1976). Loss of dendritic spines and

dendritic arborisation has been previously noted by Feldman and Dowd, (1974) and Scheibel *et al.* (1975) in the aged rat cortex. Spine loss due to ageing affects an individual neuron, rather than all the neurons. No selective reduction of any particular spine type occurs and the variability in loss is not well understood (Adams, 1986). Leuba (1983) noted a 50% decrease in dendritic spines between the ages of six months and two years in mice, but most of the spine loss occurred by one and half years. A significant reduction in spine density was observed (~12 and 10.5% in both basal and apical dendrites respectively) in the hippocampal (CA1) pyramidal cells of aged (27 month) rats (Nunzi, *et al.*1989). The main parameter for alterations among apical dendritic spine density was the dendritic diameter. It was found that thicker dendrites have greater spine densities than thinner dendrites and the comparative spine loss with age differed between 20% for thick, 36% for medium and 31% for thin diameter dendrites (Feldman, 1976) (Fig 1.6). Neurons with spine reduction are also likely to undergo alterations in their synaptic complement, since spines are postsynaptic specialisations. Synapses may disappear or new synapses might form on continuing spines on dendritic shafts. Cragg (1975) failed to confirm any age-dependent change in the number of neocortical synapses in human brain but in rats, Bondareff and Geinisman (1976) recorded a 27% decrease in synapses on molecular cells of dentate gyrus in 25 month old compared to 3 month old animals. In contrast, a postnatal study of synapses on hippocampal cells in rats showed significant difference in the density of synapses and postsynaptic dendritic spines between three and twenty four months, but the area of the presynaptic terminals and the postsynaptic spines decreased significantly as did the overall size of the synaptic contact zone in the older animals (Lolova, *et al.* 1989). Ageing not only affects the density of spines, their

morphology and their synaptic contacts, but it also influences the response of these structures to experimental conditions such as spatial learning (de Toledo-Morrell, 1984a; Vaughan and Cahill, 1984).

Figure 1.6: The trend in the loss of synapses (and increase in astrocytic area) during ageing (after Feldman, 1976).

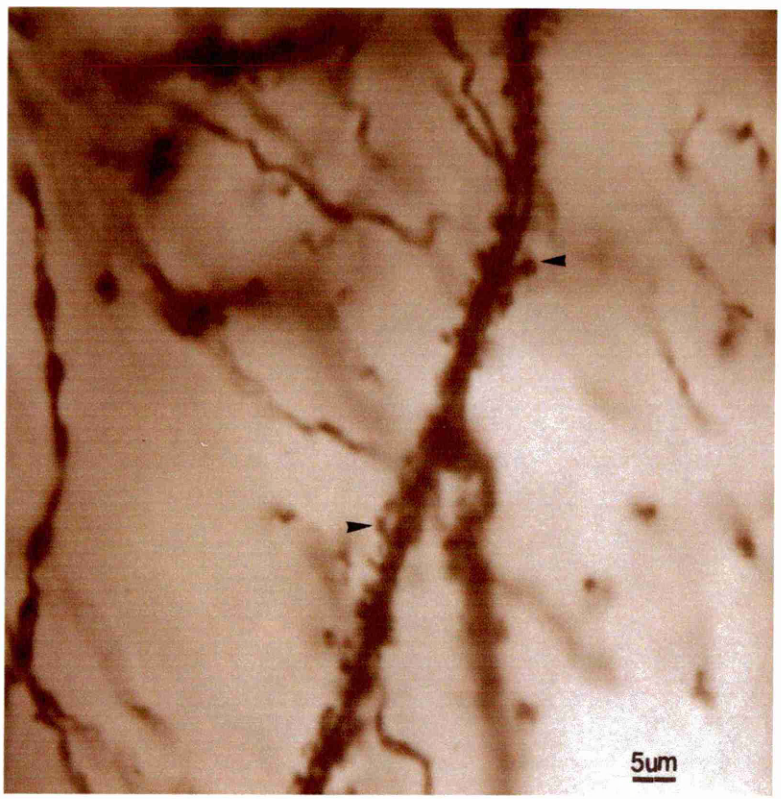


1.8.1 Dendritic Spines - Introduction:

Dendritic spines are tiny protrusions or outgrowths that stud the surface of neuronal dendrities. They vary in density both within the dendritic field of a neuron, and between neuronal types in different regions of the central nervous system (Harris and Kater, 1994). Their small size has, in large part, made them intractable to conventional experimental approaches. Their widespread consensus that dendritic spines are ideal candidates for the integration of information flow in the brain and their ability to alter synaptic morphology could be associated with learning and memory has motivated extensive efforts to obtain quantitative descriptions of spines in both steady state and active conditions (Yuste and Denk, 1995; Shepherd, 1997). Since the discovery by (Golgi, 1878) of the technique of silver impregnation and staining of approximately 10% of cells within the central nervous

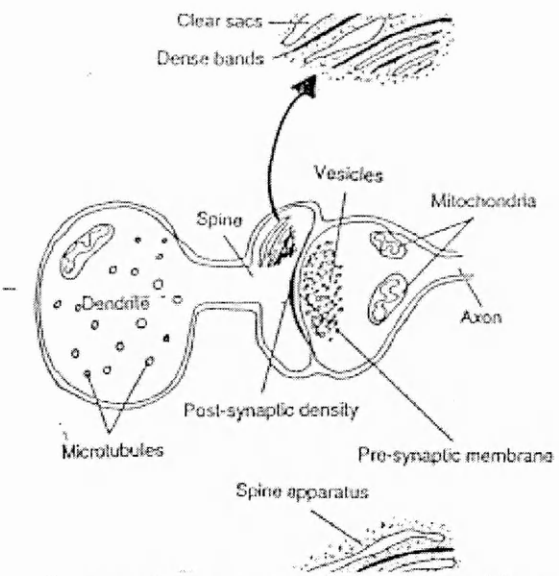
drawings of these protrusions admitting their definite existence as real structures in normal healthy adult cerebral cortex (Fig 1.7).

Figure 1.7: A Golgi stained Dendritic fragments with spines (from a rat aged 4 months).



Although dendritic spines vary in shape and size (see section 1.4.2), they are commonly described as having a head carrying a synaptic contact and a relatively narrow stalk, connecting with the dendrite (Fig 1.8).

Figure 1.8: A diagrammatic representation of a type I (asymmetrical) synapse between a presynaptic bouton and a postsynaptic dendritic spine containing a spine apparatus (after Horner, 1993).



Sometimes at the site of synaptic contact of a spine with an axon there are small protrusions called spinules (Westrum and Blackstad, 1962; Laatsch and Cowan, 1966) invaginating into the axon. It has been established that dendritic spines are an essential part of the postsynaptic membrane of the many types of neurons in the central nervous system (Scheibel and Scheibel, 1968). Excitatory axo-dendritic synapses occur more than 90% on dendritic spines in the CNS (Harris and Kater,1994). For dendritic spines, highly accurate determinations of structure (and variation) are again proving to be essential for establishing a valid concept of their function (Hosokawa, *et al.*, 1995). Recent evidence about their structure and dynamics of dendritic spines could change within two minutes was reported using GFP tagged actin (Fischer,*et al.*,1998) and fluorescent dyes such as DiI in hippocampal slices and cultures respectively (Ziv and Smith, 1996), combined with considerations of theoretical implications of dendritic spine function has produced

new and exciting considerations of their role in synaptic transmission (Larson and Lynch, 1991; Koch *et al.*, 1992; Koch and Zador, 1993). A strong working hypothesis by Koch and Zador, (1993) is that the plasticity of dendritic spines sets the boundaries within which synaptic efficacy can be modulated by a diverse group of experimental and naturally occurring conditions. This has led to suggestions of their importance in the overall cellular mechanisms of the central nervous system and formation of enduring memories.

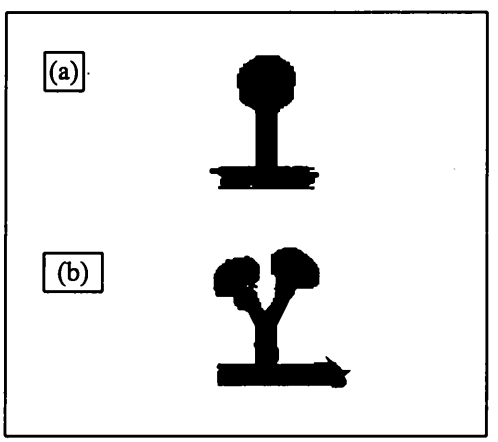
1.8.2 Location and Arrangement:

Spines are usually found protruding from dendrites, but are seldom noted on the perikaryon, axon hillock or proximal axon of neurons, where they tend to be small and sparse in number (Peters and Kaiserman-Abramof, 1970). They generally arise individually from the dendritic stem, but a few may arise from a common stalk, which bifurcates into two stalks, each bearing a spine head (Fig 1.9). Sometimes one stalk may have two end-bulbs, which arise directly from the common stalk (Peters and Kaiserman-Abramof, 1970). These spine heads may contain a spine apparatus and receive an individual axon terminal (Jones and Powell, 1969). Spine density is not consistent for all neuronal types or for all categories of dendrite. There is an understanding among researchers that there is a differential distribution of dendritic spines in relation to the different parts of the dendritic tree (Horner and Arbuthnott, 1991), but disagreement on which dendrites have the greater density of spines. There is an initial spine-free zone on apical dendrites arising from the apex of the perikaryon. This spine-free zone is followed by a gradual increase in spine numbers as the distance from the cell body increases in cortical and hippocampal neurons (Westrum *et al.*, 1964). Spine numbers reach a

maximum approximately midway along the apical dendrite in cortical pyramidal neurons and then gradually decrease in density (Peters and Kaiserman-Abramof, 1970). Golgi studies of spine distribution in hippocampal pyramidal cells describe a parabolic distribution of spines as a function of distance from the soma along branches of apical dendrites (Englisch *et al.*, 1974; Minkwitz, 1976).

The differential distribution of spines within a single class of dendrite and throughout the dendritic field is related to the extent of afferent input that the cell receives (Chan-Palay *et al.*, 1974). Methods to describe how spines are distributed along the shafts of neurons by mathematical methods have been devised (Valverde and Ruiz-Marcos, 1969).

Figure 1.9: Diagrammatic representation of branched dendritic spines.

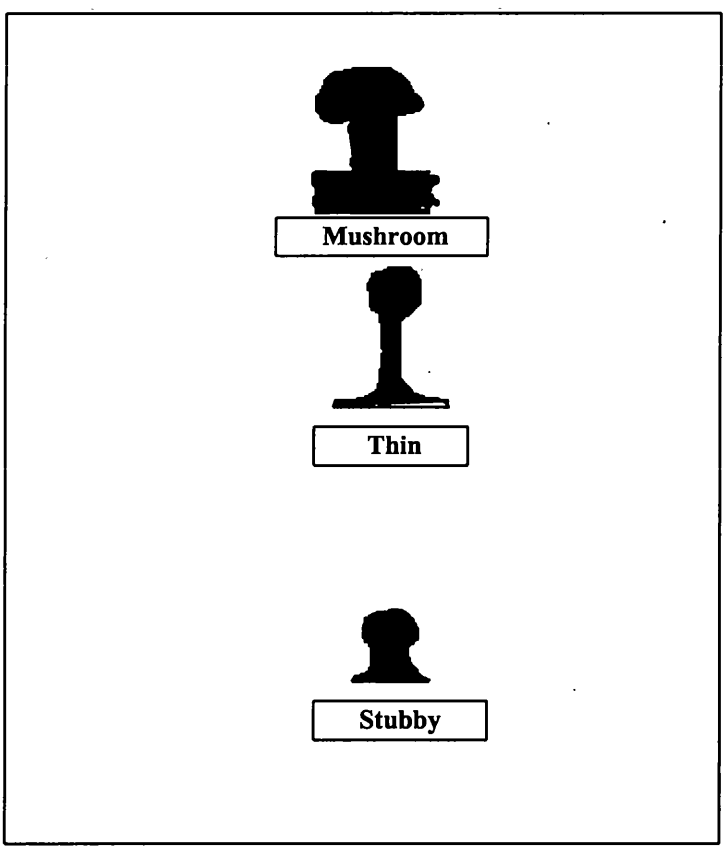


1.8.3 Shape

Dendritic spines have a variety of shapes (Peters and Kaiserman-Abramof, 1970; Crick, 1982). Although they are usually classified into three basic types: (a) stubby, which are short and thick, (b) mushroom-shaped which have a rather thick stalk widening into a bulbous terminal and (c) thin, which are slender spines that expand into a small oval or round bulb (Peters and Kaiserman-Abramof, 1970; Spacek and Harris, 1997) Fig (1.10).

Purpura (1974) found similar proportions and types of spines in human motor cortex with thin long spines noted mainly on basilar and distal apical dendrites and their branches. Variations in the morphology of spines have also been noted in the dentate gyrus (Laatsch and Cowan, 1966) where the spine density of granule cells in the rat hippocampus varies with their shape and location (Desmond and Levy, 1985).

Figure 1.10: Diagrammatic representation of different shapes of dendritic spines after (Horner, 1993).



The functional importance of different shapes or types of spines has not yet been discerned. Spines receive afferents from a variety of different sources (Laatsch and Cowan, 1966; Kawato *et al.*, 1984). However, stubby spines are the only category that appears to be restricted to a specific region of the dendritic arborization (proximal end of

the dendrites) (Peters and Kaiserman-Abramof, 1970) and populations of other spine types exist along the dendrite (Marin-Padilla *et al.*, 1969). It is possible that spine shape could be determined by the size of the axon terminal at the synapse which also suggests a dependence on the nature of the afferent input (Peters and Kaiserman-Abramof, 1970; Kawato and Tsukahara, 1984).

1.8.4 Size

Jacobson (1967) declared that spines were of relatively uniform dimension (~ 2μm long) in mammalian cortex but since then numerous Golgi studies suggest wide variations in the size of spines both within and across neurons (Crick, 1982). Whilst spines can be of all sizes on both large and small diameter dendrites (Crick, 1982), there is a strong tendency for small dendrites which are normally secondary and tertiary branches of apical and basal dendrites, to possess large spines with the longest stalks. In Golgi samples, the spines on thin diameter dendrites are of equivalent size to the dendritic diameter and therefore give the impression of being longer than those on thick main stems.

Laatsch and Cowan (1966) observed variations in the sizes of spines on dentate gyrus cells in the rat with the proximal dendrite bearing short spines. This correlates largely with the observations of Jones and Powell (1969) on cortical pyramidal spines. Dendritic spine neck diameters in the dentate gyrus area are recorded as averaging 0.13μm (Fifkova and Anderson, 1981). Similar data was observed by Wilson *et al.*, (1983) using electron microscopic spine reconstruction.

Recent studies on the hippocampal cultures by (Boyer, *et al.*, 1998) indicated an average spine length of 1.0-1.5μm and a head diameter of 0.5μm although the dimensions varied

widely between individual spines. Similar spine neck dimensions have been reported in an electron microscopic study of the rat CA1 area (Horner and Arbuthnott, 1991; Lee *et al.*, 1980). Stratum radiatum spines appear to be smaller than those described by Gray (1959a) within the visual cortex and on Purkinje cell dendrites: Tavares *et al* (1983) estimated these spines to be 1μm or less under normal circumstances. No precise variation was observed for spine dimensions in relation to either distance from the pyramidal cell layer or apical versus basilar dendritic regions in hippocampal neurons but there was considerable non-uniformity, even between adjacent or nearby spines (Turner and Schwartzkroin, 1983). The latter authors observed an average dimension of 0.22μm for neck width; 0.42μm for spine neck length and 0.72μm^2 for spine head surface area for CA3 pyramidal cells. Evidently, spines of different shapes will have different dimensions, and therefore spine size will vary both between neuronal types and within the dendritic field. The functional importance of size is debatable and not precisely known. However, since spine shape and size can be modified by various stimuli, "the state of activity" of a particular spine could influence its size.

1.8.5 Structure

As the first postsynaptic element encountered normally by excitatory neurotransmitter, dendritic spines are uniquely situated to be a fundamental integrative unit. Dendritic spines are distinguishable from most other neuronal elements by several charateristic features (Scheibel and Scheibel, 1968; Calverley and Jones, 1990; Spacek and Harris, 1997). They are deficient in the neurotubules and neurofilaments found in dendrites, but frequently contain granular material (Jones and Powell, 1969). The dendritic cytoplasms

of certain large spines are known to contain microtubules in the end-bulb (Fifkova and Van Harreveld, 1977). The floccular nature of the granular material produces a denser spinal cytoplasm than that of the parent dendrite and sometimes contains small clumps of ribosomes which could have a role in synapse construction and modification (Steward, 1983).

The synaptic strength at different spines determines the pattern of activity of individual cells and ultimately of the neuronal ensemble (Rall, 1970). Dendritic spines are so tiny and intermixed within the complex neuropil that contemporary electrophysiological and biochemical methods cannot directly assess the activity and organization of individual living spines within this neuropil. The introduction of confocal microscopy has shown that individual spines do persist over periods of several hours in hippocampal slices in vitro (Hosokawa *et al.* 1992). These observations establish an essential requirement for spines as fundamental integrative units, namely that once formed they are almost enduring structures.

Physiological data show that different excitatory synapses can have very different efficacies (Manabe *et al.*, 1992, reviewed in Lisman & Harris, 1993). If dendritic spine structures participate in defining the differences in synaptic efficacy, then the diversity of synaptic strength should be evident in spine structure. Moreover, the discrete categories might well also represent specific spine functions or stages through which individual spines must advance to achieve a "mature" state.

1.8.5.1 Spine Complex

Dendritic spines can also be considered within the context of the overall synaptic complex, which includes the spine, the postsynaptic density, smooth endoplasmic

reticulum (SER), the synaptic cleft, the presynaptic axonal bouton and its vesicle, and the neighbouring astrocytic processes. Biochemical and morphological data indicates that multiple organelles and molecules are localized within dendritic spines (Spacek and Harris, 1997).

Postsynaptic Density (PSD):

The most distinct ultrastructural feature of the dendritic spine is the postsynaptic density (PSD) (Peters *et al.*, 1991). The PSD is about 50nm thick and is opposed to the cytoplasmic side of the postsynaptic membrane. It is generally found on excitatory synapses, as well as those that occur on the heads of dendritic spines (Fig 1.11). Reconstruction of the PSDs has shown it to be either disc (macular) shaped, or highly irregular in form with perforations which have an electron lucent region within the PSD (Harris & Stevens 1989). Certain PSDs on a single spine head are segmented into distinct zones (Geinisman *et al.*, 1992). The extracellular half of the synaptic membrane of dendrite spines, in freeze-fracture preparations has an aggregate of particles, ranging in size from 6-17nm, with mean densities of about 2800 particles/μm^2 on hippocampal dendritic spines and 3600 particles/μm^2 on cerebellar dendritic spines (Harris & Landis, 1986). It has long been a theory that reorganization in the structure of the PSD reflects alterations in synaptic efficacy (Siekevitz, 1985) The molecular organization of the PSD absolutely provides numerous candidate molecules which could work in consort to negotiate the plasticity of synaptic structure and electrophysiology.

Figure 1.11: A view of postsynaptic density in the dentate gyrus of aged rats. The (stars) point to the spine apparatus (SA). The dense postsynaptic density is perforated (arrow). Magnification (× 45K)

Organelles:

Most spines contain smooth endoplasmic reticulum (SER) (Peters *et al.,* 1991, Spacek and Harris, 1997), an organelle known to be involved in membrane synthesis (Hall, 1992) and which not only stores, but also probably regulates calcium. The SER occupies about 10-20% of the total spine volume (Harris and Stevens, 1988) and is also believed to be implicated in the sequestration and intracellular release of calcium, like the sarcoplasmic reticulum of muscle cells (Hall, 1992). The inositol triphosphate (IP3) receptor has been identified on the SER in spines and dendrites (Walton *et al*, 1991). The activation of IP3

receptors by calcium in-flux, could trigger the release of intracellular calcium stored as calcium-oxalate in the SER of hippocampal and cortical dendritic spines (Fifkova *et al*, 1983).

The presence of polyribosomes has been unveiled through three-dimensional reconstruction in more than three-quarters of the visual cortical spines (Spacek, 1985b) and in at least one head of almost all the highly branched CA3 dendritic spines (Chicurel and Harris, 1992). Furthermore, polyribosomes have been found both within spines and at the base of spines in the dendrites of hippocampal dentate gyrus and CA1 neurons (Steward and Reeves, 1988). The occurrence of polyribosomes in the proximity of dendritic spines increases during synaptogenesis (Steward and Falk, 1985) and by rearing of rats in an enriched environment (Greenough *et al*, 1985). The regional synthesis of proteins could provide a cellular mechanism whereby new proteins can be marked precisely in response to synaptic activation (Steward and Banker, 1992).

Dendritic spines seldom have mitochondria, but they are generally confined to the very complex or very large dendritic spines such as those found in the branched spines of hippocampal area CA3 (Amaral and Dent, 1981; Chicurel and Harris, 1992). Likewise, multivesicular bodies are confined to large spines (Chicurel and Harris, 1992) and near the base of dendritic spines. Coated vesicles are randomly found in dendritic spines of the adult brain; their occurrence also increases with synaptogenesis, and it has been postulated that they could promote the formation of new synapses (McWilliams and Lynch, 1981).

Spinule:

The characteristic feature of most dendritic spines is a tiny projection, or invagination, of the postsynaptic terminal into the presynaptic terminal, and referred to as a spinule. In addition to the invagination into the presynaptic terminal, Routtenberg and Tarrant, (1975) observed that the spinule (i) is found inside the synaptic active zone of synapses, creating a disjointed PSD profile (that is, the spinule was located at the perforation site of PSs). It also has a constricted neck and broad bulging within the presynaptic terminal and has coated vesicles and a spine apparatus (SA) comparable with it. Furthermore, polyribosomes and rough-ER are detected close to the spinule inside the postsynaptic terminal cytoplasm; microfilaments found within the spine lie adjoining the spinule. A proposal that has received considerable attention is that the spinule may play a role in the remodelling of the dendritic spine, the synapse, or both. Despite being very theoretical, this was the earliest ultrastructurally sustained proposition that the synaptic connection may be capable of undergoing plastic change. The potential role of the spinule in the course of synaptic plasticity was subsequently reexamined by (Nieto-Sam-pedro *et al.*, 1982 and Dyson and Jones, 1984). Simultaneously, they provided stronger data confirming the proposal that the spinule could be involved in the process of synaptic remodelling and turnover.

1.8.5.2 Synapse

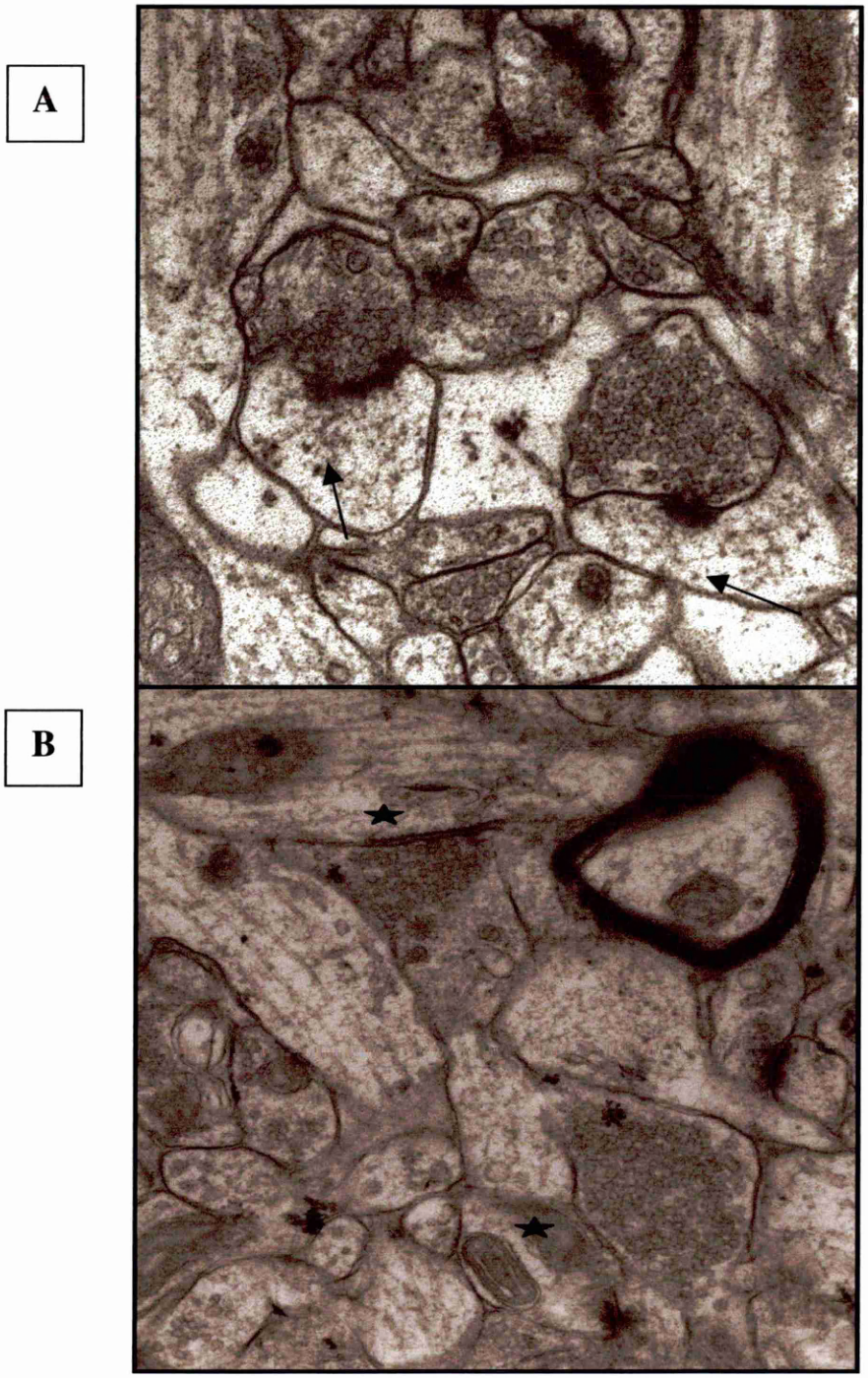
The term synapse, although coined to describe electrophysiological interactions, is referred to the specialized contact between the extension of one neuron, either a dendrite or axon, and the membrane of another cell, as viewed with morphological techniques. The mechanism of interaction is usually by means of chemical transmitters (Shepherd,

1979). The presynaptic process contains abundant synaptic vesicles and often mitochondria. The connecting regions between pre- and postsynaptic components shows localized density increases and thickenings (Westrum *et al.*, 1964), with special adhesive properties (Gray, 1959b).

Colonnier (1968) classified synapses into asymmetrical and symmetrical types (Fig 1.12). The asymmetrical synapse has a postsynaptic membrane bordered on the cytoplasmic side by a dense, thick opacity and subsynaptic organelle. But the symmetrical synapse has no dense, compact, cytoplasmic opacity different from the membrane. Asymmetrical synapses are thought to be excitatory, while symmetrical synapses are inhibitory (Raviola and Raviola, 1967).

Generally each spine has one synapse with one axon terminal from extrinsic afferents, the aymmetrical synapse being on the end-bulb (Crick 1982). Sometimes, a spine can synapse with two or more axon terminals, the stalk portion of the spine being the second contact area for the synapse (Jones and Powell, 1969). The dimensions of the synaptic junction between the spine and the axon terminal are changeable depending on the size of the end-bulb. Thin stubby spines have tiny end-bulbs with simple junctions of one or two postsynaptic densities. Mushroom-shaped spines have wide junctions with three or four postsynaptic areas, the axon terminals often bulging into the end-bulb (Peters and Kaiserman-Abramof, 1970).

Figure 1.12: The different types of synapses. Micrograph A: show asymmetrical spine synapses (arrows), while (B) shows symmetrical axodendritic synapses (stars) magnification × 45K & × 36K.



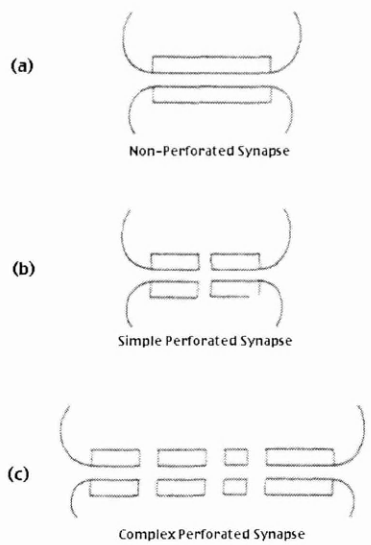
1.8.5.3 Perforated Synapses

Perforated synapses (PSs) were first observed by (Peters and Kaiserman-Abramof, 1969) in their study of synapses on dendritic spines in rat parietal cortex. Many neurobiologists considered perforated synapses to be artefactual and disregarded them, although micrographs of perforated synapses were published prior to this definitive description (Colonnier, 1968). While the size of axospinous synapses changed with the size of the two contributing components, the most conspicuous feature of the postsynaptic element was always the PSD. In the simplistic synaptic case, where the dendritic spine is tiny, the PSD spreads along the entire length of the synaptic junction. Moreover, in synapses formed by larger spines, the PSD profile was disjointed, having two or more PSD segments of varying length; this gave the appearance that more than one synapse was present (Peters and Kaiserman-Abramof, 1969; Genisiman, *et al.*, 1987, 1993; Jones and Harris, 1995). The function of perforations is to increase the perimeter of the PSD, and also the size of the total synaptic active zone. The perforations increase the efficacy of neurotransmission, if the synaptic vesicles release their transmitters at the periphery of the PSD (Jones and Harris, 1995). On the basis of the degree of presynaptic specialization synapses are divided into (i) non-perforated and (ii) perforated were further subdivided into two types as illustrated (Fig 1.13).

Further, large PSs with more complex multifarious perforations were more extensive than those having only single perforations (Geinisman, *et al.*, 1992b). It was speculated that perforations functioned to enlarge the perimeter length of the PSD, and consequently the size of the total synaptic active zone, when the diameter of the PSD was greater than

0.5μm (Jones and Calverley, 1991). Perforations were believed to increase the efficiency of neurotransmission (Buchs and Muller, 1996).

Fig 1.13: Diagrams depicting (i) a non-perforated synapse; (ii) a small simple perforated synapse; and (iii) a larger complex perforated synapse.



1.9 Cytoplasmic Organization

Initial studies on the cytoplasmic organization of dendritic spines were carried out in Purkinje cells of the cerebellum and three sets of filamentous structures were found assciated with the synaptic junction (Landis and Reese, 1983). A meshwork of filaments (5-7 nm) extends throughout the spine neck and the parent dendrite, except at the synaptic junction (Landis, *et al.*, 1987). The postsynaptic membrane was found to be associated with a web of filaments (4-6nm) and larger microfilaments, possibly actin (8-10nm), extend into the spine. The shape of the spine depends on the organisation of these filaments (Markham and Fifkova, 1986, Jones, 1975).

The contractile protein-actin, has been localised in hippocampal neuons and dendritic spines (Drenckhahn, *et al.*, 1984; Moshkov, *et al.*, 1985; Fischer, *et al.*, 1998). The

preferential expression of actin in certain synaptic areas; such as dendritic spines and presynaptic terminals, suggests an important role in altering spine shape and synaptic curvature (Drenckhahn, *et al.*, 1984; Cohen, *et al.*, 1985; Moshkov, *et al.*, 1986). During development actin expression is highest in dendrites and spines and the expression increases as spines became more complex (Markham and Fifkova, 1986). The membrane associated cytoskeletal protein - Calspectrin- was detected only during development in dendrites and spines, but was not found in adults. Thus, calspectrin could play a role in synaptic plasticity during development (Kimura, *et al.*, 1987). Fifkova and Morales (1989) suggest that calcium-regulated contractile and cytoskeletal proteins could control synaptic plasticity of dendritic spines.

1.10 Calcium Control

The large electrical resistance provided by the narrow spine neck constriction has been the focus of theoretical investigations into spine function. A massive diffusional resistance is caused due to the narrow spine constriction. The activation of calcium currents in spines, results in a peak spine head calcium ion concentration which is greater in "long, thin" spines rather than mushroom or stubby spines (Holmes, 1990).

A biophysical model combining electrical and calcium ion activity accounts for the phenomenology of LTP induction at a Hebbian synapse in the hippocampus (CA1) (Zador, *et al.*, 1990; Sejnowski, 1997) (see chapter 2). The calcium-dependent synaptic modification based on computer simulation proposed the following:

(1) Compartmentalising transient changes in calcium concentration to specific synapses satisfying the conjunctive requirements for synaptic modifcations.

(2) Isolating changes in calcium concentration at the dendritic shafts rather than the spine heads.

(3) Activated synapses show amplified calcium concentrations.

(4) The voltage-dependent process underlying LTP induction is increased.

It has been observed that short high- frequency bursts of presynaptic activity are more effective in raising the calcium levels, especially the calcium-calmodulin complex, rather than sustained low frequency activity (Gamble and Koch, 1987). Therefore, the ability of spines to concentrate calcium ions could play a role in LTP induction.

Chapter 2.0

Long-term Potentiation (LTP) and Synaptic Plasticity

" Aging is a natural process that must be studied intensively, for it remains one of the most agonizing problems in all biology. Not only must the gerontologists continue to concentrate on performing the research necessary to understand aging, but they must assume an increasing role in the application of their knowledge for betterment of the status of the aged."

(Lawton, 1965:31)

2.1 Long-term Potentiation:

Neurobiologists believe that information or substrate for "memorytraces" is encoded in the brain by changes in the synaptic strength between neurons (Maren and Baudry, 1995). The excitatory connections made by the perforant path fibers onto granule cells of the hippocampus, a cortical region necessary for the formation of memory in man, is the first structure were synapse was identified in the mammalian brain. The efficacy of synaptic transmission is increased abruptly and held by brief trains of high-frequency stimulation to monosynaptic excitatory pathways in the hippocampus (Bliss and Collingridge, 1993). The discovery of a long-lasting increase in synaptic efficacy following electrical stimulation of the rabbit hippocampus, was termed long-term potentiation (LTP) (Bliss & Gardner-Medwin, 1973). Long-term potentiation (LTP) is characterized by three basic properties of cooperativity, input-specificity and associativity (Bliss and Collingridge, 1993) is gradually being considered to constitute strong evidence favouring the hypothesis that this form of synaptic plasticity is probably a biological substrate for learning and memory, still universal acceptance for this hypothesis requires experimental evidence. Recent experimental evidence has shown that saturating LTP impairs spatial learning in two separate learning tasks (McNaughton, *et al.*, 1986; Castro, *et al.*, 1989). These studies conclude that both LTP and spatial learning rely on the similar cellular mechanisms. However, the above data has been contradicted by others who have failed to substantiate these findings carried out recently (Jeffrey and Morris, 1993). Ageing studies have indirectly shed light on the fact the LTP could be a biological substrate for certain forms learning is significant by the evidence that indicates that both spatial learning and LTP are compromised in aged rats (Lynch, 1998).

Since its discovery long-term potentiation (LTP) has been found in all excitatory pathways in the hippocampus, as well as other brain areas have been shown to support similar forms of plasticity (Rogan, *et al.*, 1997) (Fig 2.1a,b).

Figure 2.1a: The Hippocampal Extracellular Field Potential: this represents a Population of Synchronously Active Neurons; the Potential is termed the Excitatory Postsynaptic Potential (EPSP) (From Teyler and Discenna, 1984).

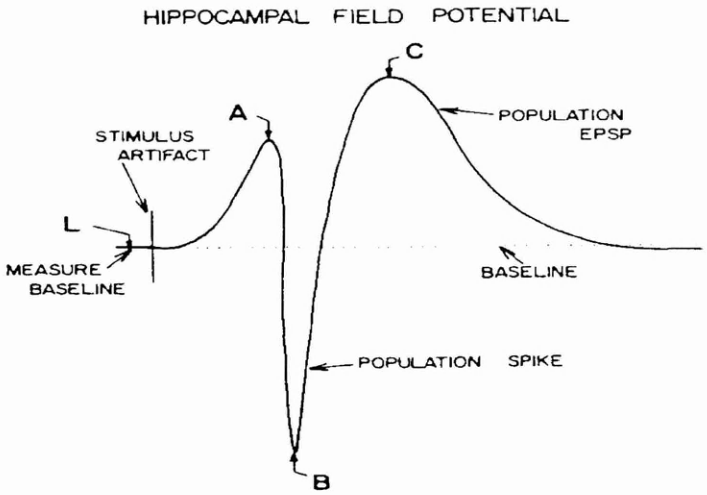
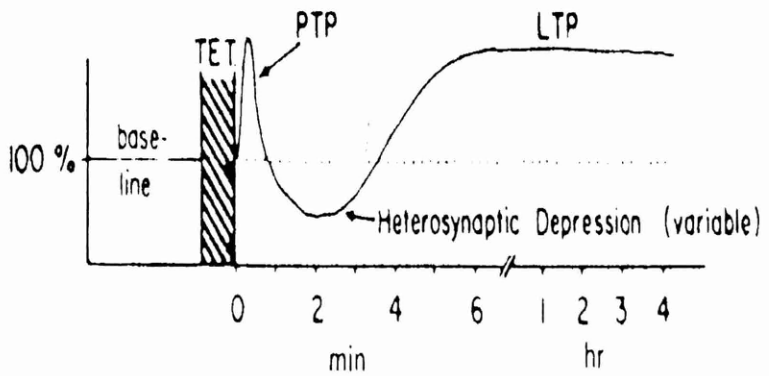


Figure 2.1b: Shows the Properties of Long-term Potentiation (LTP) (from Teyler and Discenna, 1984).



LTP in the hippocampus is becoming a prominent model of activity-dependent synaptic synaptic plasticity in the mammalian brain, and progress is being made to elucidating the mechanisms underlying its induction and expression (Collingridge and Bliss, 1995). A fundamental characteristic of LTP, which is utilized in hippocampal studies, is an elevation in the excitatory post-synaptic potentiation (EPSP). The population EPSP should be at least 20% above the baseline, for the animal to be considered to have sustained LTP induction. It can be induced in numerous ways, most suitably by delivering a tetanus (typically a train of 50-100 stimuli at 100Hz or more) to the pathway of interest (Maren and Baudry, 1995). The most intensively studied forms of hebbian plasticity is LTP in the hippocampus, that results from coincident pre- and postsynaptic activity. This long-term form of activity-dependent synaptic plasticity can be differentiated from less enduring forms of synaptic plasticity such as short-term potentiation (STP) and post-tetanic potentiation (PTP), by its cellular mechanism. STP and PTP persist for minutes following induction, while LTP lasts for hours and even days (Douglas and Goddard, 1975; Geinisman, 1994; Kullman, 1992; Harris, 1984).

Properties of LTP:

A considerable amount of research is being directed towards understanding the biochemical, molecular and synaptic mechanisms of hippocampal LTP. A hallmark example of hebbian synaptic plasticity is hippocampal LTP; simultaneous pre- and post-synaptic activity yields a persistent increase in the efficacy of synaptic transmission. The hebbian nature of LTP arises from the specific activation requirements of NMDA receptors. The existence of an intensity threshold for induction descirbes co-operativity; for example 'weak' tetanic activating relatively few afferent fibres, do not trigger LTP

(McNaughton, *et al.*, 1978). The threshold phenomenon of LTP induction is a complex function of intensity and pattern of tetanic stimulation; between 'strong'trainsthat induce LTP and 'weak' trains that produce post tetanic potentaition (PTP). LTP is input-specific, since inputs that are not active at the time of the tetanus do not share in the potentiation induced in the tetanized pathway (Lynch, *et al.*, 1977). Finally LTP is associative in the sense that a 'weak' input can be potentiated if it is active at the same time as a strong tetanus to a separate but convergent input (Levy, *et al.*, 1979).

One of the most important properties of LTP is associativity because it provides a cellular analogue to learning (Brown, *et al.*, 1990). For example; during conditioning an initial neutral conditioned stimulus (CS; i.e. the weak pathway) comes to elicit a conditioned response (CR) similar to the unconditioned response (UR) elicited by an initial nonneural unconditioned stimulus (US; i.e., the strong pathway). Hence, in the above example, depolarization generated by the strong US pathway promotes NMDA receptor activation and LTP in the weaker CS pathway. Finally, these phenomena provide strong support for the involvement of LTP in learning and memory (Kelso, *et al.*, 1986).

LTP has been shown to exhibit properties similar to memory (Berger, 1984). For instance, LTP can be induced rapidly (it attains steady state in 10 minutes after induction) and once estabished it is quite resistant to disruption. Hippocampal LTP *in vivo* lasts for hours to several days depending upon the parameters and stimulation pathway. Furthermore, LTP can be disrupted prior to stablizing by a variety of manipulations (electroconvulsive shock, hypoxia and cooling shock etc) (Staubli& Lynch, 1990; Fujii *et al.*, 1991). The liability of LTP for disruptions indicates a consolidation period

frequently observed in behavioral studies of learning and memory (Zola-morgan & Squire, 1990).

A number of groups have reported that saturation of LTP impaired spatial learning in two different spatial learning models (McNaughton, *et al.*, 1986; Castro *et al.*, 1989). They concluded that both LTP and spatial learning relied on similar cellular mechanisms. However, this data has been suggested to be inaccurate by others who haven't been able replicate these findings in a more recently (Jeffrey and Morris, 1993). More recently Moser *et al.*, (1998) after lesioning a large part of the hippocampus, used two bipolar stimulating electrodes implanted in the dentate gyrus, and saturated a substantial proportion of the perforant path synapses in the dorsal hippocampus on the intact side. It was then observed that tetanized animals were impaired in water maze task. This lends support to the earlier work by McNaughton *et al.*, (1986); Castro, *et al.*, (1989). Inspite of these differences, it is appropriate to dwell on the similarities in the cellular/molecular mechanism reponsible for spatial learning and for the induction, maintenance of LTP. The above information further supports the hypothesis that LTP is a probable biological substrate for learning and memory.

2.3 Long-term Depression (LTD)

Certain patterns of neuronal activity can also lead to persistent decreases in synaptic strength, or long-term depression (LTD). Long-term depression of parallel fibre-Purkinje cell synaptic transmission, initially observed in the rabbit (Ito *et al.*, 1982), was confirmed in cerebellar slices excised from the guinea pigs (Sakurai, 1987). Like LTP, LTD has been documented in many brain areas, including visual cortex, the

hippocampus, and the cerebellum (Ito *et al.*, 1989). In this *in vitro* preparation, excitatory synaptic potentials were recorded intracellularly from the Purkinje cell following stimulation of parallel fibres. When stimulating the parallel fibres concurrently stimulated the climbing and parallel fibers at 4Hz for 25 sec, the amplitude of EPSP's evoked in the Purkinje cell was depressed for more than 60 minutes. Repetitive stimulation of either the climbing fibre or the parallel fibre alone at the same frequency did not depress the EPSP's; instead, it produced a moderate potentiation lasting 10-50 min (Sakurai, 1987). The observed decrease in synaptic strength is believed to result from a reduction in the sensitivity of postsynaptic AMPA receptors (Linden *et al.*, 1991).

But unlike LTP induction, the critical events in LTD induction involve the coupling of a potent Ca^{2+} signal generated by climbing fiber discharges with activation of mGluRs at parallel fiber-Purkinje cell synapses. Thus, cerebellar LTD does not involve NMDA receptor activation (in fact, adult Pukinje cells lack NMDA receptors), but an increase in intracellular Ca^{2+} in postsynaptic Purkinje cells is required (Sakurai, 1988). This elevation in intracellular Ca^{2+} is probably mediated by both voltage-gated Ca^{2+} channels activated by climbing fiber depolarization and the liberation of intracellular Ca^{2+} stores by a metabotropic receptor-mediated second messenger cascade (Okamoto & Sekiguchi, 1991). Thus, the final common pathway for the induction and expression of both hippocampal LTP and cerebellar LTD is an elevation of intracellular Ca^{2+}, an activation of enzymatic cascades, and a modification of postsynaptic AMPA receptors.

2.3.1 Long Term Potentiation and Dendritic Spines:

The dominant rationale for a functional description of spine morphology has been the desire to comprehend the morphological substrate of the intense synaptic plasticities seen

in the hippocampus and cortex. The most extensively examined paradigm, is long-term potentiation (LTP), described earlier. Despite the ongoing debate over the precise series of events during LTP (whether pre or postsynaptic), an increasing agreement holds that alterations in the properties of both the pre- and postsynaptic elements are implicated (Kullman and Nicoll, 1992; Bliss and Collingridge, 1993).

2.3.1.1 Possible Role of Dendritic Spines in LTP Induction:

LTP induction requires the entry of calcium into the postsynaptic bouton (Collingridge and Bliss, 1987). To accomplish this calcium entry at the majority of synapses where LTP is induced, glutamate should be discharged from the presynaptic terminal at (or near) the same time that the postsynaptic element is depolarized. The postsynaptic depolarization is essential to release a magnesium block in the calcium channel that is integral with the N-Methyl-D-aspartate (NMDA) receptor (Madison *et al.*, 1991). Change in morphology of dendritic spines, e.g contraction of the neck, would result in augmentation of the depolarization accomplished in the close proximity of the synapse, relative to that which would be generated if the synapse occurred directly on the dendritic shaft (Brown *et al.*, 1988) (Fig 2.2).

Figure 2.2: A dendritic shaft synapse (Axodendritic Synapse) in the middle molecular layer of the dentate gyrus of aged rats (arrow). Magnification (×45)

Data from ontogenetic studies on rat hippocampus support assumptions about the importance of spines and synapses in LTP. Immediately after birth, no potentiation is elicited from tetanic stimulation in area CA1, but by postnatal days 3-4 post-tetanic potentiation, persisting less than a minute, can be induced (Harris and Teyler, 1984). By days 5-7 a more persistant potentiation can be induced, which lasts for only 45 minutes post tetanus (Bekenstein and Lothman, 1991). By days 10-11 the potentiation endures for 2.5 hours, and by day 15 some animals show enduring LTP (for at least 9 hours in vitro). These observations cannot be explained easily by the development of NMDA receptors: within area CA1, NMDA receptors are represented at about 75% of adult values from birth through day 7 (McDonald *et al.*, 1990). The outgrowth of a minimum number of

dendritic spines might be required for the induction of LTP, as spines are initially present at days 5-7, and during this time a nonpersistent type of LTP is first induced (Harris *et al.*, 1989). Remarkably, with further development more spines contain constricted necks, and LTP can be induced at lower stimulus intensities than those necessary at the younger ages when there are fewer spines (Harris and Teyler, 1984). Spines could likewise promote the efficiency of the maturing NMDA receptors and ontogeny of LTP in the cortex (Tsumoto, 1992).

2.3.1.2. Modifications in Dendritic Spine Structure Following LTP:

The dentate gyrus is composed of granule cells. The total of number of granule cells in a single rat dentate gyrus is approximately 0.6×10^6 (Amaral et al.,1990), and appears to depend on the age (Bayer et al., 1982) and strain of the animal. In part, the variability in cell counts is due to the proliferation of the granule cells well into adult life (Bayer et al., 1982). The summed length of all the dendrites of an average granule cell in the suprapyramidal blade is approximately 3500μm, whereas the infrapyramidal blade has a dendritic tree of approximately 2800μm according to Desmond and Levy (1982,1985). Trommald, (1990) estimated the number of dendritic spines on the granule cell dendrites, the cells in the suprapyramidal blade had 1.6 spines/μm, whilst the cells of the infrapyramidal blade had 1.3spines/μm.

Normal spine and synaptic measurements have been compared in preparations, which have undergone experimental plasticity, along with those which have not. Many other reviews have considered the alterations in spine morphology that accompany synaptogenesis during development, behavioural alterations associated along with

learning and memory, and pathological changes associated with neural dysfunction (Calverly and Jones, 1990). Evidence strongly suggests that changes in spine and synaptic structure occur during LTP (Wallace *et al.*, 1991). On the ultrastructural level, researchers have recorded changes in spine and synaptic morphology that have been explicitly linked to the tetanized input (Fifkova and Van Harreveld, 1977 and Desmond and Levy, 1988a). Disagreement remains as to whether new spines and synapses form or if the geometry of existing spines and synapses changes (Harris *et al.*, 1992). For instance, in the dentate of the hippocampus, some data indicate that dendritic spines swell during LTP (Fifkova and Van Harreveld, 1977), whilst others suggest an alteration in the morphology of existing PSDs during LTP (Desmond and Levy, 1990). Nevertheless, results from an investigation applying serial EM reconstruction suggest that during LTP the total spine number doubles in the dentate and the number of branched spines and spines with wide necks increases (Trommald *et al.*, 1990). Within hippocampal area CA1, no notable changes in overall spine density were found following LTP induction, but there was confirmation of spine "rounding" and an increase in the occurrence of stubby dendritic spines (Chang and Greenough, 1984).

As suggested by the report of dendritic spine organization earlier (chapter 1), several molecular mechanisms exist that might cause swift short-term and long-term changes in spine and synaptic morphology. For instance glutamate and its analogues initiate proteolysis of brain spectrin (fodrin) by the neuron-specific protease, calpain I (Siman and Noszek, 1988). Degeneration of fodrin, a fundamental protein of the (spine) cytoskeleton (Permutter *et al.*, 1988), could induce the spine to undergo shape changes (Siman *et al.*, 1990), probably in response to synaptic growth. Actin polymerization is

controlled by calcium concentration and determines the coherence of the spine cytoplasm (Fifkova and Morales, 1989; Holmes, 1990). The actin filaments are temporary structures that may modify quickly in response to the calcium-activated second messenger systems involving stimulation of phosphorylation of calmodulin (Kimura, *et al.*, 1987). Actin could promote alteration in spine structure via its coupling to the subplasmalemmal cytoskeleton or may alter spine structure through "contraction", recently it has been shown that spine motility is a robust and ongoing feature of dendritic function using GFP-actin and time-lapse video microscopy (Eccles, 1983; Fischer, *et al.*, 1998).

2.3.2 Long-term Potentiation and Synapses:

Considerable research has been devoted to determining whether LTP also has a structural correlate at the synaptic level (Wallace, *et al.*, 1991; Edwards, 1995). Evidence for alterations in synapse number with LTP in the CA1 region initially came from analysis by Lynch and his co-workers (Lee, *et al.*, 1981). They demonstrated that electrical stimulation, which produced LTP either in vivo or in vitro, led to a swift increase in the number of synapses onto dendritic shafts.

Chang and Greenough (1984), found a rapid (within 10 -15 min after stimulation) increase in the number of shaft synapses per unit area and an increase in the number of sessile spine synapses (assumed to be immature or transient synaptic connections) in potentiated slices, compared to those from corresponding low-frequency stimulated naïve groups.

Alterations in synapse number and/ or structure in the dentate gyrus following stimulation of the perforant path have been reported to occur as quickly as 2-30 min following

induction of LTP (Fifkova and Van Harreveld, 1977; Fifkova, 1982; Desmond and Levy, 1990; Trommold, *et al.*, 1990), and persisted for periods from hours to days (Geinisman, *et al.*, 1991, 1994; Rusakov, *et al.*, 1997). Whilst earlier studies used single-section analyses (biased counting, see below) Chang, *et al.*, (1991); Buchs and Muller, (1996) utilized biased methods (Gundersen, 1986) to estimate the number or structure of hippocampal CA1 synapses after electrically or chemically induced LTP. Variability in synapse size, shape, or orientation significantly influences the probability of synapses being viewed on a single section (Sterio, 1984; Braendgaard and Gundersen, 1986; Coggeshall and Lekan, 1996). Thus, such "older" methods have been termed "biased", even though the authors most certainly didn't intend this to be the case.

2.3.3 The Aims of This Thesis:

Normal human aging is associated with selective changes in cognition that are attributable, in part, to dysfunction of hippocampal pathways (Squire, 1992). Rodents also exhibit hippocampal dysfunction that results in spatial memory deficits (Zornetzer, 1982; Zelinski, 1988). Long-term potentiation (LTP) is widely studied as a paradigm of synaptic plasticity, and is considered as a probable synaptic model for learning and memory. Few data exist from use of modern unbiased stereological methods exist on the morphological changes in dendritic spines and synapses at early time points (45 min) after LTP Induction. It is unclear whether morphological changes occur in hippocampal synapses at this time post-LTP, and if so, whether they occur to the same extent in young and old animals.

The objectives were therefore:

1. To determine whether synaptic and dendritic spine changes occur post-LTP in aged rat hippocampus.
2. To examine if morphological alterations are either a result of, or a pre-condition of, LTP in aged animals.
3. To compare morphological changes following LTP in young and aged animals.

Chapter 3

General Methods

3.1 Animals Used:

Male, Sprague-Dawley aged n=8 (22 months) and young n=6 (3 months) rats bred at (Trinity College, Dublin) weighting 250-800 gms, served as experimental animals. Dr. M. Lynch or A. Kelly carried out all electrophysiology. The animals were anaesthetised with sodium pentabarbital solution (30-40mg/kg injected intraperitoneally) and fastened in a stereo-taxic apparatus. Recording and stimulation electrodes made of lacquer insulated tungsten wire were lowered to stimulate the medial perforant path, while the recording was done more rostrally from the dentate molecular layer.

The coordinates for the recording electrode were 5.2mm behind the bregma and 4.3mm lateral to the midline. For the stimulating electrode they were 7.2mm behind the bregma and 2.8mm lateral to the midline. The signals unique for dentate granule cells in response to stimulation of the medial perforant path were used to locate the position of the recording electrode. Since the EC-DG system is largely unilateral (Steward and Vinsant, 1983), each animal served as its own control, i.e., one side of the brain received brief, high-frequency conditioning stimulation (the stimulated side) and the contralateral side did not (the control side).

3.2 Electrophysiological Method:

Long-term potentiation was induced by tetanic stimulation of the perforant path (3 trains of 250Hz for 200 millisec). This test shocks (1/30sec were delivered for 10 min before, and 45 min after tetanic stimulation). The response to test shock stimulation before tetanization was used as baseline and long-term potentiation was measured as an increase in the slope of the EPSP. The degree of LTP was assessed by comparing two input/output

curves, one taken before, and one taken 45 min after the last tetanization. The mean increase in population EPSP slope in the last 10 min of the experiment compared to the 10 min prior to tetanization was 119% ± 2.89 SEM Fig (4.3). To insure that the research was unbiased, the animals were coded at Trinity College, Dublin and the code was not released until all the data was collected, entered, and ready for statistical analysis.

3.3 Fixation and Perfusion:

Fixation is carried is carried out in order to stabilize the cellular architecture. It prevents the tissue from degeneration due to endogenously activated enzymes or as a response to the action of microorganisms. It enables preservation of tissue components for subsequent processes. It causes hardening and strengthening of tissue to aid sectioning. The most suitable fixative is one that penetrates rapidly into the tissue, and is essentially irreversible. A double fixation method is used, where the primary fixation is by buffered aldehyde (paraformaldehyde and glutaraldehyde) solution followed by secondary, or post-fixation in 1% osmium tetraoxide. The aldehydes stablize the tissue by crosslinking to proteins, while osmium tetroxide preserves unsaturated lipids, and stains the tissue block black, providing contrast at the electron microscope level.

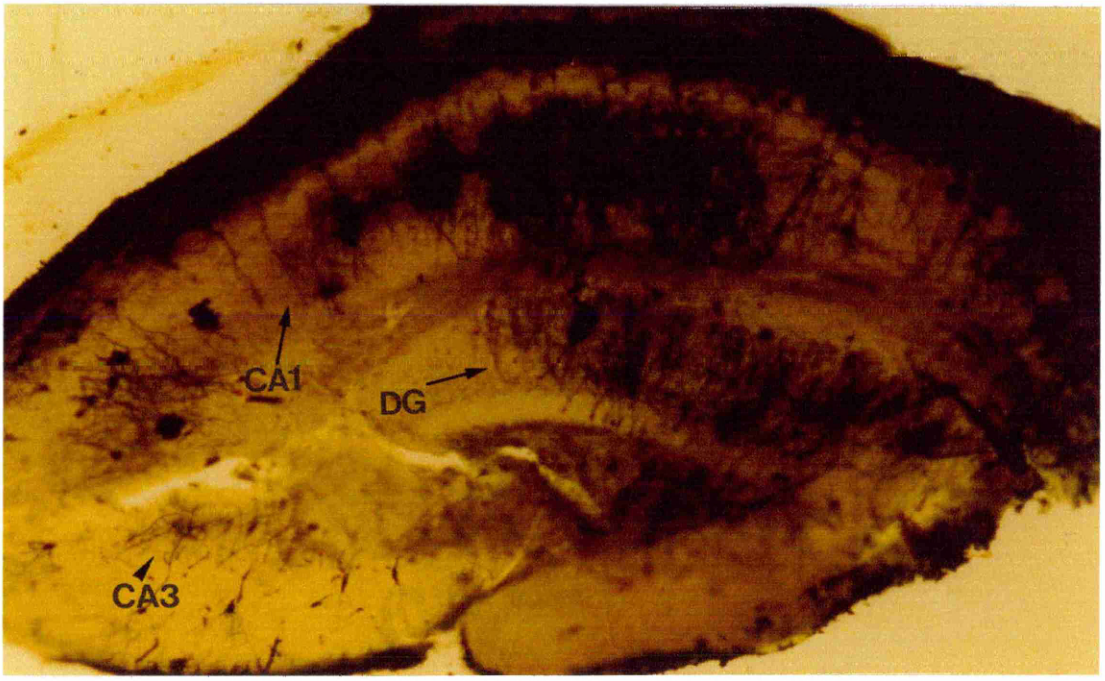
At the end of the recording period animals were dissected to expose the heart and the pericardium is removed. A needle was inserted through the left ventricle after ligating the left aorta. Once the needle was secured in position, approx 30ml of 0.9% saline was pumped (via a peristaltic pump) at room temperature (21^0C) to drain the blood from the brain through the punctured right atrium. Gradually the saline was replaced with 2% paraformaldehyde and 2% glutaraldehyde for 30 minutes. After fixation the brain was

dissected out and left overnight in fixative. The hippocampal formation was taken out and two or three 1mm thick, transverse tissue slabs were cut, one slab for Golgi impregnation and the adjoining slab for electron microscopy. The control tissue was processed in the same way, from the contralateral hemisphere.

3.4 Golgi Impregnation:

The rapid Golgi method described by (Fairen *et al*, 1977) allows the dendritic spines to be studied and characterized both at the light microscopy and electron microscopy level. Impregnation by the Golgi method reveals tremendous insight into the three dimensional nature of the neurone and its processes. It is the most widely used method for the morphological characterization of neurons and in evaluating loss of dendritic arborisation in neurones of the cerebral cortex in degenerative diseases and dementia (Scheibel, 1978) (Fig 3.1).

Figure 3.1: A Golgi Impregnated Hippocampal Section. Shows the cornu ammonis (CA fields) and dentate gyrus (DG).



The unexplained phenomenon, which makes the method so useful, is that only 10% of the total neuronal population become impregnated, giving a clear picture of neuronal architecture uncluttered by surrounding cell processes. The duration of incubation in 1% osmium tetroxide (OsO_4) affects the density of stained neurones. The longer the incubation in osmium tetroxide, the darker staining appears. A discontinuous impregnation of neuronal processes occurs along with artefactual deposits, if the staining is improper and obscures structures of interest.

3.5 Embedding for Golgi:

The tissue blocks were washed in 0.1M phosphate buffer, pH=7.3 for 15 minutes, then transferred to 0.5% osmium tetroxide in 7% glucose, 0.003% $CaCl_2$ in 0.2M phosphate buffer, pH = 7.3 for 4 hours. The blocks were washed in 8g glucose + 30ml standard buffer + 0.2ml 1.5% $CaCl_2$ + 65 ml distilled water, before storing in 3.5% potassium dichromate for 48 hours, later the tissue slabs were washed in used silver nitrate, in order to remove the precipitate caused by the reaction between silver nitrate and potassium dichromate and finally the tissue slabs were stored in fresh 0.7% silver nitrate overnight in complete darkness. The time recommended for the impregnation can vary depending upon the required end result, generally with any Golgi method, optimal times can only be determined by experience.

The tissue slab was superficially embedded in 7% agar and 100-120μm thick was cut using a Sorvall tissue chopper. The tissue was kept wet with glycerol. At this stage the sections were mounted on glass slides for examination under the light microscope.

Finally the sections were dehydrated in alcohol, cleared in xylene and embedded in DPX mountant for examination in a Zeiss axiophot light microscope.

3.6 Embedding for Electron Microscopy:

After fixation the brain was dissected out and left overnight in fixative before dissecting the structure of interest. The tissue slabs were washed in 0.2M standard buffer (NaH_2PO_4/ Na_2HPO_4), pH = 7.4 for 15 min and then transferred to 1% osmium tetroxide in 0.1M phosphate buffer for 4hrs. Later the blocks were rinsed in washing medium (8g glucose + 30ml standard buffer + 0.2ml 1.5% CaCl + 65ml distilled water) and dehydrated in a graded series of acetone.

Dehydration:

- 30% Acetone 10 min
- 50% Acetone 20 min
- 70% Acetone 20 min
- 90% Acetone 20 min
- 100% Acetone 3 times 15 min
- 100% Acetone + molecular sieve 10 min

While in the final stages of dehydration, ingredients for Epon, DDSA and MNA are warmed for 20 min to 60^0C. In order to prepare 20 ml of Epon 812, 16 ml of DDSA and 8ml of MNA are well mixed. BDMA (1.3ml) is added subsequently and the amalgam is well mixed again in a mixing container and then gently by hand. Due to the toxic nature of the vapours, embedding media are handled in a fume cupboard.

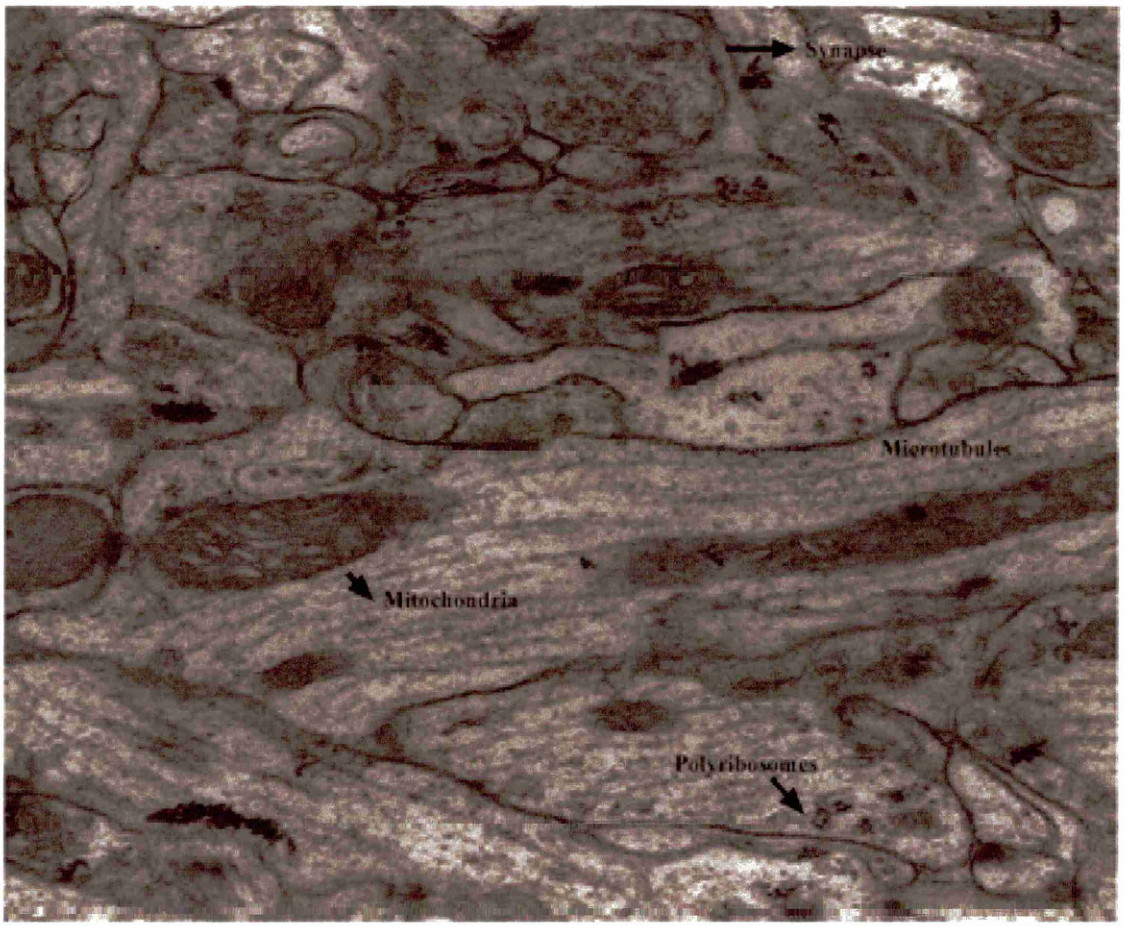
Following dehydration the specimens were left soaking for infiltration with the embedding medium until the dehydrating agent has been completely replaced by the final embedding mixture.

Procedure:

- 1:1 Epon/Acetone overnight on rotating mixer with the bottle caps off,
- Fresh Epon 3hr on rotating mixer with the bottle caps off, and
- Finally Epon 2 hr on rotating mixer with the bottle caps on.

Sections are placed at the bottom of capsules and filled with freshly prepared resin and allowed to polymerise at 60^0C overnight. After polymerisation, the blocks are cut on an ultramicrotome using glass and diamond knife at thickness of approximately 80nm and mounted on piloform carbon coated slot grids. The sections can be stained with uranyl acetate (Leica Ltd, England) for 50 min at 35^0C and lead citrate (Leica Ltd, England) for 10 min at 20^0C in an automatic LKB Ultrastainer. The piloform carbon slot grids are coated with carbon, in order to prevent the movement of section due to heat generated by electron bombardment. The carbon coated grids with the serial sections are loaded into the electron microscope and visualized at 80KV current. An example of an hippocampal dendritic ultrastructure is shown below in (Fig 3.2).

Figure 3.2: Electron micrograph of the Hippocampal Dentate Gyrus (Middle Molecular Layer). A dendritic shaft (Den) synapses. Mitochondria (mit), smooth endoplasmic reticulum (SR), microtubules (m), small aggregates of ribosomes (r), pre-and postsynaptic boutons are visible.



3.7 Estimation of Hippocampal Shrinkage during Fixation:

Hippocampal tissue shrinkage in electron microscopic preparations was estimated. Hippocampi were dissected from three adult rats. 300μm thick transverse hippocampal slices (the thickness was chosen to satisfy embedding protocol conditions) were cut using a Mcllwain tissue chopper while being watered with cool Hanks solution. Twelve slices (four from each animal) taken from the middle third of each hippocampus, were selected for analyses, arranged individually on glass slides, and imaged using an MCID image

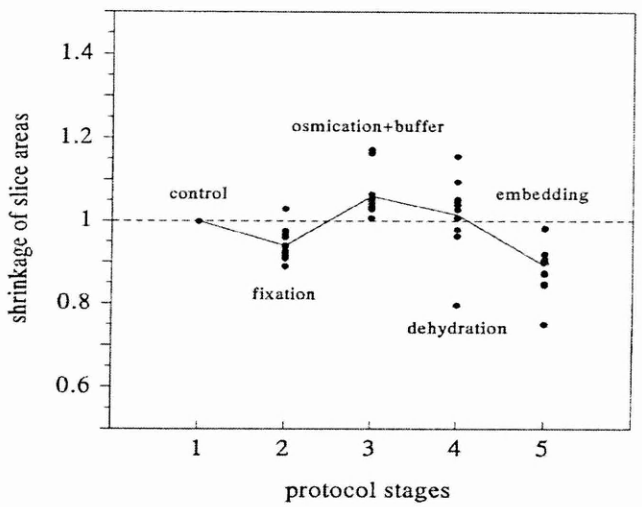
analysis system (Imaging Inc.) via a high resolution video camera. At subsequent stages, images of each individual slice were captured systematically while the slice was post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon 812 exactly as described earlier (Doubell and Stewart, 1993). To prevent the slices from curling, osmication and dehydration were carried out while the slices were covered with a light plastic cover slip.

In slice images corresponding to each protocol stage of embedding, the visible area of the slice was measured using image analysis routines in NIH 1.55 Image. Because the slice thickness is relatively stable in each slice, final changes in the visible slice area compared with the acute (fresh) slices, represented unbiasedly the squared linear shrinkage/expansion. Two of the twelve slices developed a noticeable crack in the final stages of the protocol suggesting the possibilty of small potential shrinkage anisotropy in some hippocampal preparations during embedding.

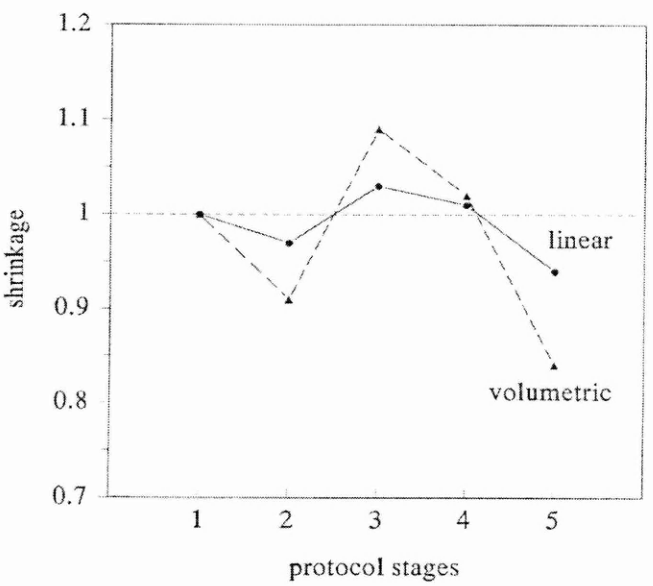
These measurements have been shown that the tissue volume of the embedded hippocampal specimen is 84.3 ± 2.8%, and the linear/distance measures are 94.3 ± 1.1% (mean ± S.E.M.) of those in fresh (acute) preparations. The shrinkage obviously did not affect any of the relative measures determined in our study, whereas absolute linear or volumetric quantities shown below account for the shrinkage values (Fig 3.3 a, b).

Figure 3.3 a, b: Shrinkage of hippocampus during fixation process.

A



B



3.8 Image Analysis of Dendritic Spines:

3.8.1 Hardware: Light Microscopy

Morphometric analysis of dendritic spines was performed on Golgi stained hippocampal sections. Impregnated dendritic fragments were examined, captured and stored for further

analyses using a Nikon Microphot Microscope equipped with Bosch video camera, linked to a Magiscan MD Image Analysis System (Applied Imaging, Sunderland, UK). The images of interest were captured at a magnification of × 500 (Fig 3.4).

Dendritic fragments, normally 0.8-0.9μm in diameter, were sampled in the middle third of the molecular layer, approximately 70-80μm from the cell body layer (that is, the terminal zone of medial perforant path synapses since the perforant pathway was stimulated in the present experimental paradigm). The sampling was at random, moreover fragments examined and captured were approximately parallel to the plane of view of the microscope. Approximately 16-18 dendritic fragments, 10 -25μm long, were analyzed. The images captured were stored on 128Mb optical discs.

Figure 3.4: The Magiscan MD Image Analysis System used for morphometric analysis on Golgi impregnated hippocampal sections.

3.8.2 Hardware: Electron Microscopy

Neuromorphometric analysis of synapses was carried out using a JEOL 1010 transmission electron microscope (Fig 3.5). High resolution images (1317 × 1035) were acquired directly using a kodak megaplus camera attached to the 35 mm port of the microscope column and stored on (128Mb) magento-optical disc using a Macintosh Quadra 950 desktop computer. The JEOL 1010 microscope is equipped with an opto-mechanical interface device from Advanced Microscopy Techniques (AMT) of Rowley, MA, USA. It comprises two major assemblies, the phosphor screen and mirror which is moved in and out of the beam, by a retracting mechanism, and is mounted on the right-hand side of the column at the 35 mm camera port. The camera and lens are mounted on the fixed assembly found on the left-hand side of the column. It is essential that the images projected by the phosphor screen and mirror is aligned with the camera, as a fraction of one pixel misalignment will cause significant distortion. The JEOL 1010 is equipped with a stage controller. In the 'disector' technique its essential to store the area of one section, before moving to the second section that is 80nm further into the block.

Figure 3.5: JEOL 1010 transmission electron microscope used in the morphometric studies on aged and young rats after LTP induction. A Kodak CCD-TV camera is attached to the column of electron microscope, which allows images to be transfered to Macintosh Quadra 950 microcomputer for storage on magneto-optical disc, using NIH 1.55 IMAGE software.

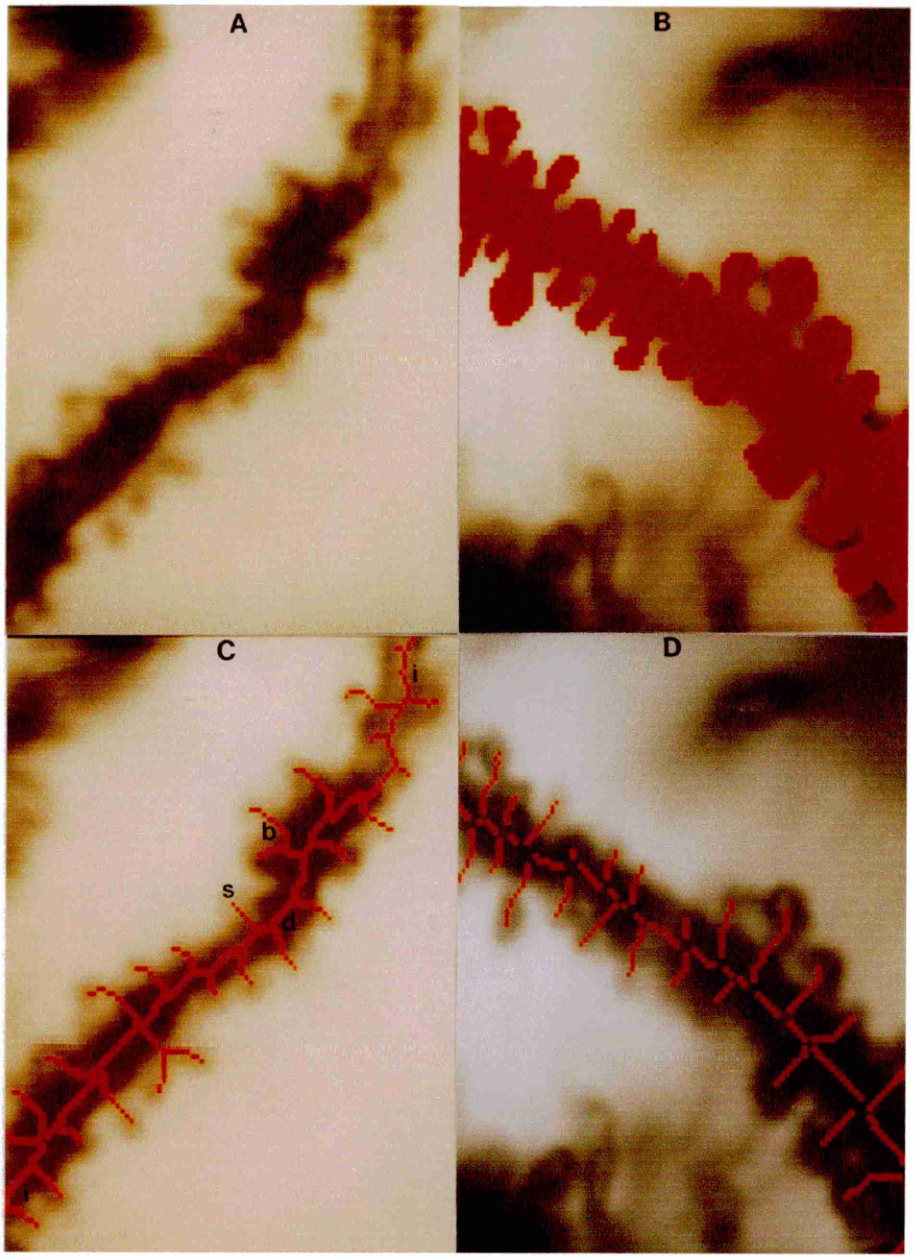
3.8.3 Light Microscopy: Software

The software used for the morphometric analysis was "Genias 4.3" (Applied Imaging, Sunderland, UK.). The contour of the perceivable dendrite was resolved by applying an object-background partition alogrithm (Rusakov and Stewart, 1995). The intervention of the operator is required in determining the dendritic profile while the computer automatically does the remaining decisions regarding spine measures.

3.8.3.1: Parameters for Branched spines, Spine Length & Spine Density:

The binary profile of the dendritic fragment is established (Fig 3.6 a, b, c, d), using an approved mid-axis transformation, the binary image was skeletanized automatically (Rusakov and Stewart, 1995). We selected the mid-axis as a relevant measure of the spine length since (a) its curvilinear length reflects the visible spine shape, and (b) it measures from the mid-"axis" of the dendrite to the "mid-point" of the spine head, hence providing a better credible spine length for those spines whose parts are hidden in the shade of the dendritic shaft. The shape of the spine influences this method of measuring spine length: rounder spines will normally yield shorter length measures than a thin linear spine projecting a similar distance from the dendritic border. The observed spine length, inter-spine distances and percentage of branched spines were automatically sorted by the software and stored in a file. While spine density was estimated by the mathematical formula: 1/ inter-spine distance (Rusakov and Stewart, 1995).

Figure 3.6: Quantification of dendritic-spine structure using image analysis operations. A: representative picture of a dendritic fragment (middle molecular layer of the dentate gyrus). B: Threshold image of the dendrite (shown in red). C: Line skeleton obtained from the threshold image. D: Measured lengths: notations: s, spines; d, inter-spine distances along the dendrite; b, branched spines; i, ignored. Scale bar A-D: 8μm.



3.8.4 Software: Electron Microscopy

Images were acquired, processed and manipulated using NIH Image 1.55 software that allows advanced processing capability. The software permits image enhancement and background subtraction that was used in the present study.

The Perceptics PTDCI frame grabber board (Pixel Tools Digital Camera Interface) located in the Macintosh Quadra 950 (33MHz)-microcomputer aids in acquiring images on a 16" monitor. A systematic random sampling procedure was used to collect 24 images at an electron microscope magnification of ×8,000, which is approximately × 30, 000 when viewed on the computer screen. Images acquired comprised almost 1.4 MB of information, hence they were stored directly on to 128 Mb magneto-optical discs.

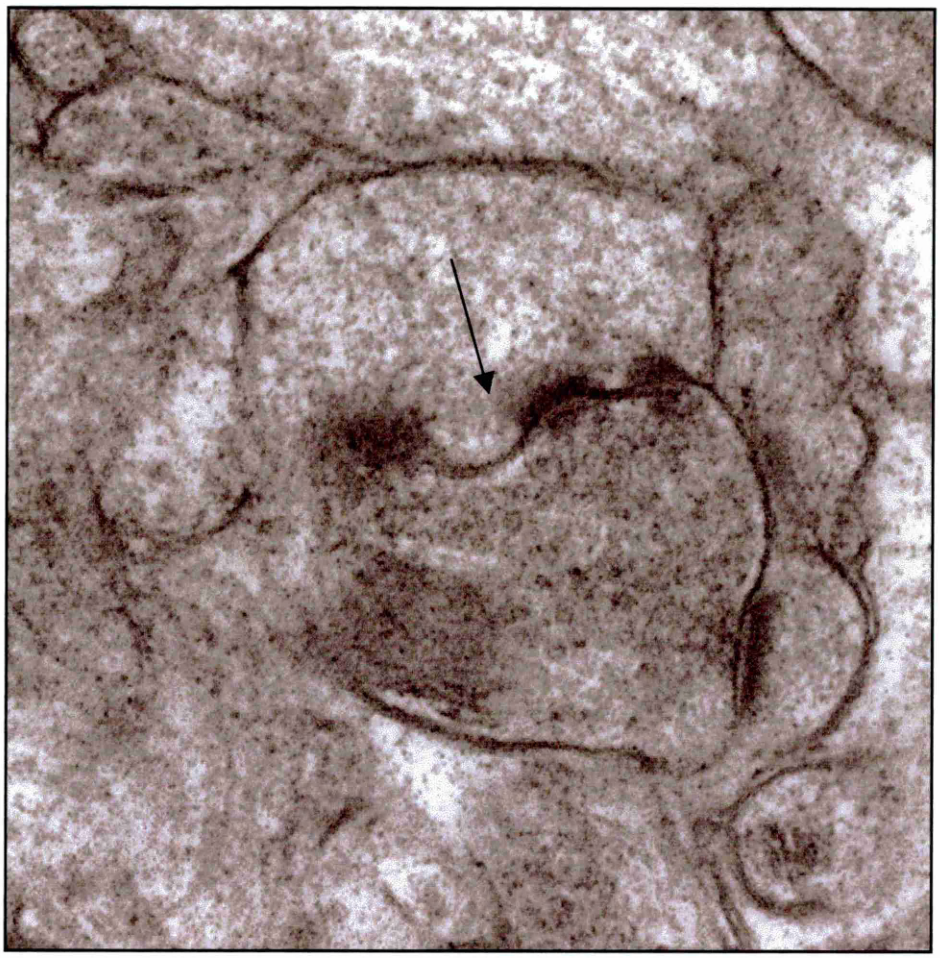
Images that were acquired on section A, were stored on the stage controller, later the stage was moved to the adjacent section B, and the corresponding position was located and stored. The position on section A was then automatically recalled using the stage controller. Synaptic density was analyzed by recalling image pairs and drawing windows (18 × 18 cm, equivalent of 35μm) and the two pairs were viewed side by side on a 20" monitor on a Macintosh Quadra 950 computer system.

3.8.4.1: Parameters for Complex Vs Simple Perforated Synapses:

The fundamental premise of learning and memory is that something must change in the structure of the neuron for long term memories to be stored. The focus of this thesis was to look at the changes in synaptic density and percentage of perforated synapses following LTP induction. The criteria for identification of simple perforated synapses

from complex perforated synapses are shown diagrammatically in (Fig 1.13). An example of a complex perforated synapse is illustrated in (Fig 3.7).

Figure 3.7: Complex perforated synapse in the middle molecular of the dentate gyrus 45 minutes after LTP induction. Magnification ×45K.



3.9: Statistical Methods:

Data were analysed by one-way ANOVA and by Students 't' tests (dependent and independent). Results were considered to be significant at $P<0.05$.

Chapter 4

Correlation between Dendritic Spine Morphology, Synaptic Density, and the Ability of Aged Rats to Sustain LTP in the Dentate Gyrus, 45 Min after Tetanic Stimulation of the Perforant Path

4.1 Introduction:

Anatomical studies on the normal ageing human brain usually show a decrease in the number of synapses with increasing age (Scheibel, *et al.*, 1976; Pupura, 1974; Flood, *et al.*, 1987; Einstein, *et al.*, 1994). In the precentral gyrus this decrease has been shown to be due to a decrease in asymmetrical axospinous synapses (Adams, 1987). However, symmetrical synapses stay consistent in number, although axodendritic synapses show a small increase with age (Adams, 1987). Along with the decline in synaptic number is an increase in mean length of the postsynaptic contact zone (Adams, 1987). Hence, the human brain is capable of synaptic plasticity in response to ageing-induced synaptic loss, and this plasticity could be a compensatory response by the remaining synapses to age-related synaptic loss.

The importance of the hippocampus in memory formation (O'Keefe and Nadel, 1978; Olton, 1983; Squire, 1988), its role in spatial learning (Morris, *et al.*, 1986; Morris, *et al.*, 1990; Dunnett, 1992), and the use of LTP in the rodent hippocampus as a model of synaptic plasticity, was discussed in earlier chapters. Interestingly, aged animals are deficient in the maintenance of LTP (Barnes, 1980, McGohan, *et al.*, 1997). While they can be potentiated to the same extent as young rats, they lose enhanced synaptic responses more swiftly (Barnes and Mc Naughton, 1985; de Toledo-Morrell, *et al.*, 1988).

Quantitative electron microscopic analysis of the dentate gyrus of rats indicates ~ 30% decrease in synaptic density (of all synapses) during ageing Geinisman, *et al.*, (1976). In this area certain modifications in spine and synapse number and/or structure have been

reported to occur as early as 2-30 min after induction of LTP (Fifikova and Van Harreveld, 1977; Desmond and Levy, 1990; Trommald, *et al.*, 1990), and to have lasted for hours to days (Geinisman, *et al.*, 1991, 1994; Rusakov, *et al.*, 1997) see section 2.3.1.2 & 2.3.2). A confocal microscopy study of living hippocampal slices has provided evidence for changes in spine length and mobility following chemically induced LTP (chemLTP) (Hosokawa, *et al.*, 1995). Confocal microscopy also has shortcomings. It does not identify short or curved dendritic spines from the protruding dendritic shaft nor the occurrence of shaft synapses, and it also lacks sufficient resolution to measure synaptic dimensions (Trommald, *et al.*, 1995).

A recent, quantitative study of the distribution of dendritic spines in hippocampal granule cells, suggest a dense cluster of spines surrounding the dendritic stem termed 'spine collars' (Rusakov, *et al.*, 1995), and a reduction in spine density after 24 hrs of unilateral LTP induction (Rusakov, *et al.*, 1997). These facts suggest that the pattern of axospinous synapses and spines along the dendrites affects the spatio-temporal summation of multiple synaptic inputs (Rose and Call, 1992), and indicates that the arrangement of spines along dendritic branches might reflect important properties of local synaptic circuitry that could influence LTP.

4.1.1 Aim:

The present experiments aim to addresses the question of whether deficiency in LTP induction within aged animals (potentiated and non-potentiated) has a structural substrate at the level of dendritic spines and synapses. Here, we used rapid Golgi staining (Fairen, 1977), unbiased image analysis (Rusakov, *et al.*, 1995), tilting disector (Rusakov, 1995), serial sections (DeHoff, 1982; Mayhew, 1992), and the unbiased 'disector technique'

(Sterio, 1984; Gundersen, 1988), which allows quantification of the distribution of spines and synapses along the dendrites. The results provide precise measurements of alterations in spine density, spine length, percentage of branched spines, shaft synapses, spine synapses, synaptic density and percentage of perforated synapses along dendritic branches of the granule cells in the middle molecular layer of the dentate gyrus of aged (22 month old) rats 45 minutes after LTP induction. A preliminary report of these data has already been published (Dhanrajan, *et al.*, 1997; Stewart, *et al.*, 1997; Dhanrajan, *et al.*, 1998).

Methods, perfusion, fixation and procedure for light and E.M. microscopy are described in the preceding chapter 3, and details of buffers and chemicals are given in the appendix.

4.2 Methods:

4.2.1 Measurement of Dendritic Spine Length and Spine Density

Spine length is defined as the (curvilinear) distance from the mid-point of the dendrite to the middle of spine head. Whilst dendritic spine density can be assessed by several methods, until recently, spine density was estimated relatively simply by counting the number of visible spines along a length of dendrite, using an ocular micrometer (Equ. 1) (Valverde, 1967). Numerous studies have reported spine densities of apical, basal and oblique or transverse and terminal tuft dendrites in heterogeneity of cell types. This kind of estimate is an effective indicator of spine density within the same dendritic type of the same cell type from the related species. However, various geometrical equations have been designed in an attempt to overcome the underestimation, which results from using visible spine counts and omits hidden spines out of the plane of focus. These stereological

corrections are based on assumptions, such as, restrictions in the size, shape, and/ or spatial layout of spines and dendritic stems (Equ 2-4).

Equation 1:

$$\text{Dendritic spine density} = n / Dl$$

Where, n= number of visible spines, Dl = dendritic length over which spines are counted.

Equation 2: (Feldman and Peters, 1979)

$$N = n\pi[(Dr + Sl)^2 - (Dr + Sd)^2]/$$

$$\{\theta/90\pi(Dr + Sl)^2] - 2[(Dr + Sl)\ Sin\theta\ (Dr + Sd)]$$

Where, N = an estimate of the total number of spines, n = number of visible spines, Dr = dendritic radius, Sl = spine length, Sd = diameter of the spine head, $Cos\theta$ = Dr +Sd/ Dr +Sl, Dl = dendritic length over which spines are counted. Therefore: Dendritic spine density = N/Dl.

Equation 3: (Chan-Palay, *et al.*, 1974)

Linear number of spines = n/2

(Number of 100μm units in the total Dl)

N = Linear number of spines

(Dc/ distance between spines)

Where N = estimate of the total number of spines, n = visible number of spines per 100μm segment of dendrite, Dl = dendritic length assessed, Dc = Dendritic circumference.

Equation 4: (McConnell and Berry, 1978)

Correction factor = $(2r \times \text{arc cos } (r/r +)^{-1}$.

4.2.2 Measurements and Principle of Tilting Disector Method for Dendritic Spines:

Recently, a conventional image analysis process, based on modified stochastic geometrical methodology, and a design based stereological method has been developed by Rusakov, *et al.*, (1995) which allows an unbiased primary quantification of big populations of dendritic fragments viewed at the light microscopy level. Spine length and the distances between spines along the dendritic stem, are the two sets of raw data that can be quantified using this procedure, therefore depicting a foundation for further analyses. This procedure enables an unbiased sterological assessment of the true (3D) numerical density of spines along dendrites, and has been termed "the tilting disector", a technique which utilizes design-based stereology (Gundersen, 1986; Mayhew, 1992).

4.2.3 Measurements and Principle of Tilting Disector Method for Dendritic Spines:

The fraction of visible and true numbers of spines detected through the light microscope relies on their size, form, and spatial arrangement, moreover on the breadth of the dendritic column. Hence, computation of precise spine density on the dendrites has been a significant issue in the area of neuromorphology, and as discussed above various stereological correction factors have been proposed (Chang-Palay, *et al.*, 1974; McConnel and Berry, 1978; Feldman and Peters, 1979; Koch, 1992).

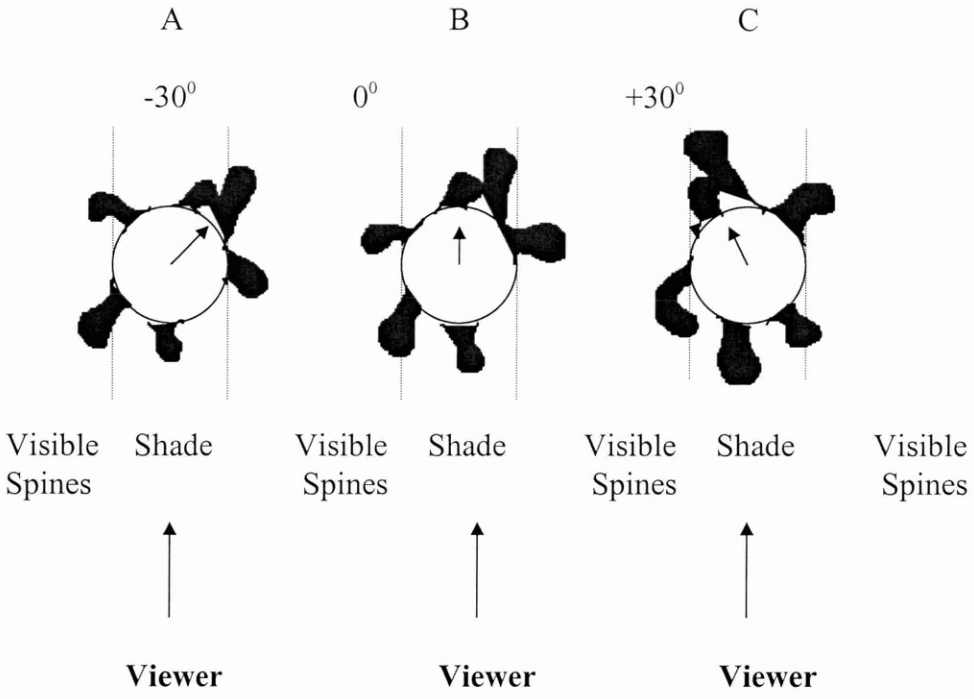
The central approach lies in the design-based stereological procedure called the disector (Sterio, 1984), that excludes the obstacle of statistical presumptions about the structures of interest, in which the 3D density of random particles is computed directly, by estimating the particle 'edges' detected in pairs of juxtaposed sections of accepted thickness (Gundersen, 1986).

The only assumption required in the present approach of estimating the true spine density along dendrites is that the distribution of spines around the longitudinal dendritic axis is even, which is plausible in the majority of cases. An unbiased estimate is performed by simply scoring the number of spines within a known angular sector (in the present experiment middle molecular layer of the dentate gyrus) around the dendritic longitudinal axis. Evidently, the total number of spines N (are scored within a 15^0 sector of β degrees around the dendrite axis), the estimated total number of spines (i.e., within a 'sector' of 360^0) N is

$$N = 360n/\beta$$

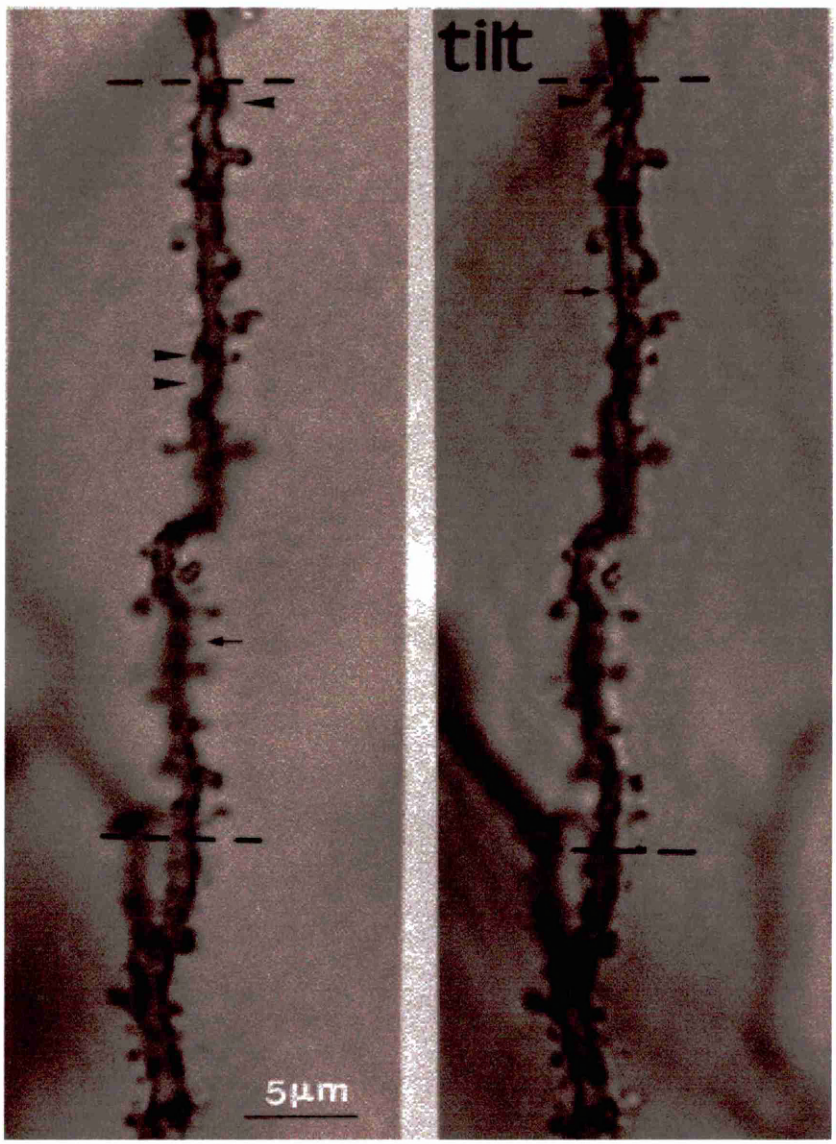
The dendritic fragments are rotated around their longitudinal axis for a certain angular sector using the tiling device, in light microscope preparations. The procedure of counting the number of spines (on a dendritic fragment) within a sector of tilting/ rotation is comparable to that of the disector routine; only visible spine are counted in one angular position. Hence, each spine was associated with a particular reference (Rusakov, *et al.*, 1995). (Fig 4.1a,b)

Figure 4.1a: A schematic cartoon of the dendritic cross-section illustrating counting procedures in the tilting disector (from Rusakov and Stewart, 1995).



In the above cartoon A-C: 3 different angular positions (of 30^0 each), of the same dendritic fragment, with respect to the viewer. Dotted lines border the area in sight of a viewer; circled '+' and '–' signs represents spines present in one angular position and absent in the other angular position.

Figure 4.1b: A Golgi Dendritic Fragment using the Tilting Disector Technique. Frame A: The arrow points to visible spines, Frame B (after tilting): The arrow points to the appearance of a new spine.



A **B**

4.2.4 Estimation of Thickness of Ultrathin Sections:

The "electron scattering" technique, was used for the estimation of section thickness 't'.

Electron scattering is resolved directly in the electron microscope by measuring the electron current on the viewing screen with the exposure meter. The differences in electron scattering between the section on the support film ES(s) (an area of 'empty' resin, which is a blood vessel) and support film ES(f) on the other hand. The measurements were carried out at an accelerating voltage of 80KV and an electron microscope (JEOL 1010 see below) spot size of 2. The difference between the two regions was used to calculate the relative number of electrons transmitted by the section (de Groot, 1988).

$$R.E.T. = [ES(s)/ES(f)] \times 100$$

$$\text{'t' Thickness} = (97.465 - R.E.T.) / 0.4739$$

4.2.5 Measurement and Principle of Disector Method for Synapses:

Synapses are observable only under electron microscope due to their relative small size, hence estimation of synaptic density can only be made on a two-dimensional plane which intoduces bias as described earlier (Chapter 2&3). In fact, several authors have reported changes in number, size and shape of synapses most likely reflecting the synaptic function. It is, therefore essential to use an accurate unbiased method to estimate synapses (Sterio, 1984, Gundersen, 1986, 1988; Mayhew, 1992).

In the past, morphological estimates of particles in 3-D used biased estimation based on assumptions, regarding spatial orientation and synaptic shape. Moreover, synapses have been known to deviate from the assumed disc-shape (De Groot, 1985, 1988). Synapses can be 'complex-shaped', 'curved' or 'perforated' and the probability of them hitting a

plane more then once is high, leading to difficulties in deciding whether or not they belong to a single synapse. Formulas used to estimate numerical densities (Nv) are:

$Nv = Na / d + t$..... (Abercrombie, 1946)

$Nv = Na.t / (4d/\pi) + t-2h$..... (DeHoff and Rhines, 1961)

$Nv = Na / d$..... (Colonnier and Beaulieu, 1985)

In which Na is the number of synapses per unit, t is the thickness of the section, h is the height of synapses and d the mean profile diameter. The significant drawback is to take into consideration the "mean caliper diameter" d, which is influenced by orientation, size distribution and particle shape. Hence, biased methods should no longer be applied in morphometric estimations (Cruz-Orive and Weibel, 1990).

Stereological methods such as the 'disector' (Sterio, 1984) and the 'fractionator' (Gundersen, 1986) have succeeded due to their superior efficiency. The 'disector' technique was applied in all the experiments reported in this thesis. It is independent of any assumptions about shape, size and orientation of the synapses quantified.

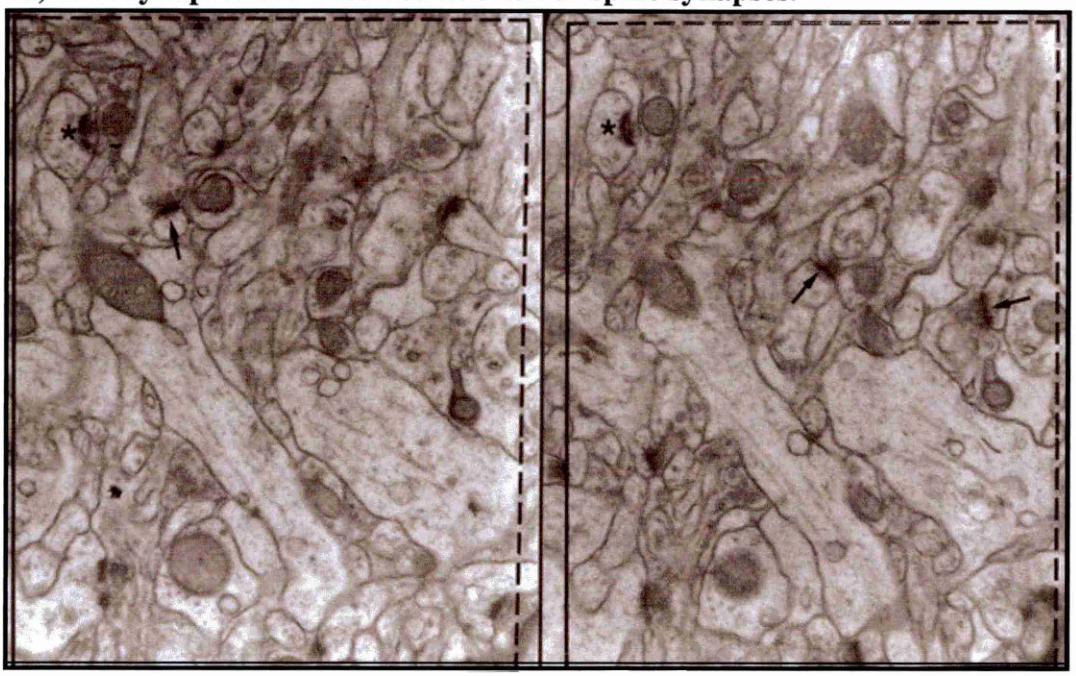
The principle is based on unbiased 3-D rule, synapses are counted if they appear in an unbiased counting frame on one slice (the 'reference') but not on its adjacent section (the 'lookup') (Fig 4.2). Synapses that don't cross the two fully drawn edges and those not present in the adjacent serial section are counted (N syn).

Accordingly the mean synaptic density $N\nu_{syn}$ can be calculated as follows:

$$N\nu_{syn} = \Sigma \frac{N_{syn}}{tA}$$

In which 't' is the thickness and 'A' is the area of the counting frame.

Figure 4.2: A Disector Frame. Synapses are selected using a three-dimensional probe, in which two micrographs of the same fields of view (A and B) are taken from adjacent sections. The synapses are counted if they are located within one counting frame, but are absent in the second field (arrow). The synapses on the dotted line are not counted (arrow-head) or if they are present on both sections (stars). The synapses are classified as shaft or spine synapses.



The calibration of the measured distance is done using a grating replica, (2160 lines/ mm) and area of the counting frame was 35μm. The field of view is located on a conspicuous nuclei in a corner of the nominated section A & B. The viewing field for both the 'reference' and 'lookup' sections was selected at low magnification (8000×) and stored digitally on magneto-optical discs.

In the present experiment, from each aged rat, 24 disector images were captured from serial ultrathin sections of the left and similar number from the right hemisphere. Synaptic density ($N_{V_{syn}}$ per μm^3) for all visible synapses was estimated, without discrimination between individual types, regardless of whether spine or shaft, since 90% of the total

of the total visible synapses comprise asymmetrical synapses. Furthermore, asymmetrical synapses were classified into those occurring on dendritic shafts and dendritic spines.

4.2.6 Data analysis and statistical design:

Inter-hemispheric analysis of spine length, spine density, percentage of branched spines, density of shaft synapses, density of spine synapses, synaptic density and percentage of perforated synapses was performed using a one-way ANOVA design, which eliminates the variance between individual animals while contrasting the hemispheric difference. The estimated lower and upper 95% confidence limit was applied to the data obtained. The EPSP slopes obtained for each aged animal was correlated with the changes in spine density and spine length using a randomized block analysis of variance (ANOVA) design.

The following factors were examined:

a) hemispheric (left/right) differences, and

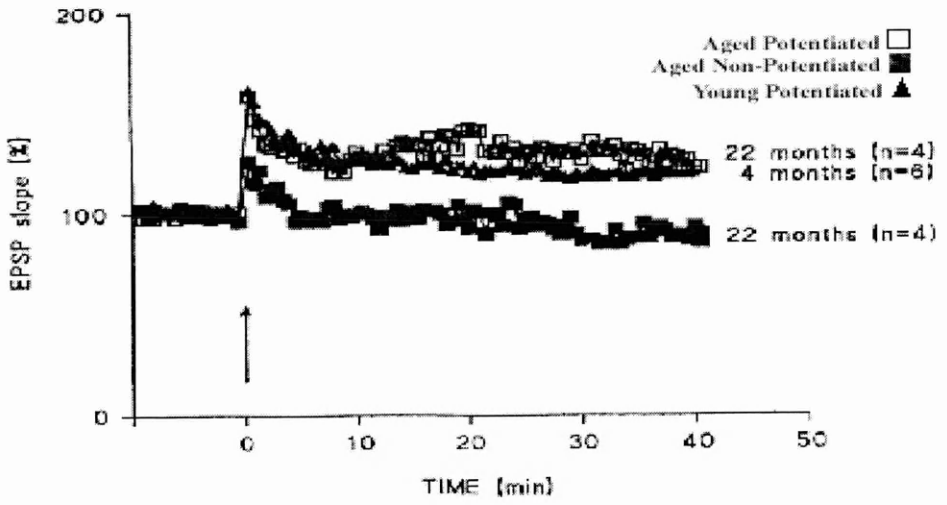
b) The effects of high frequency stimulation.

4.2.7: <u>Synaptic plasticity changes in aged rats 45 minutes after LTP induction</u>:

Long-term potentiation (LTP) was unilaterally induced in the hippocampal dentate gyrus of aged (22 month) rats. Experiments were performed in the laboratory of Dr. Marina Lynch, Dept. of Physiology, Trinity College, Dublin. A total of N = 8 aged animals were stimulated. One group of animals (N = 4) sustained LTP (maintained LTP ~20% above the baseline EPSP), while the other group (N = 4) did not sustain LTP 45 minutes after induction (showed no increase above baseline level) (Fig 4.3). Since the EC-DG system is largely unilateral (Steward and Vinsant, 1983), the left hemisphere of each animal served as its own control, i.e., one side of the brain (right hemisphere) received brief, high

frequency conditioning stimulation as described above (the stimulated side) and the contralateral side did not (the control side). These results correlate with earlier work done in the present lab and others (Trommald, 1990; Geinisman, *et al.*, 1993, 1994, 1997; Rusakov, *et al.*, 1997) (Fig 4.3).

Figure 4.3: The mean EPSP slope for aged (and young rats)* 45 minutes after LTP induction.



4.3 Results:

4.3.1 Numerical Spine Density: (Light Microscopy)

Fig 4.7 shows the changes in spine density in the middle molecular layer of dentate gyrus of aged rats 45 minutes after LTP induction. Based on the EPSPs obtained after stimulation (Fig 4.3), the aged rats were divided into two groups (N = 4) each. The aged rats that showed an elevated EPSP (approximately 20% above the baseline) were classified as potentiated and those that showed no elevation in EPSP as non-potentiated (Fig 4.3)

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* Data for young rats relates to experiments described in chapter 5 and is shown here for comparative purposes relating to that work.

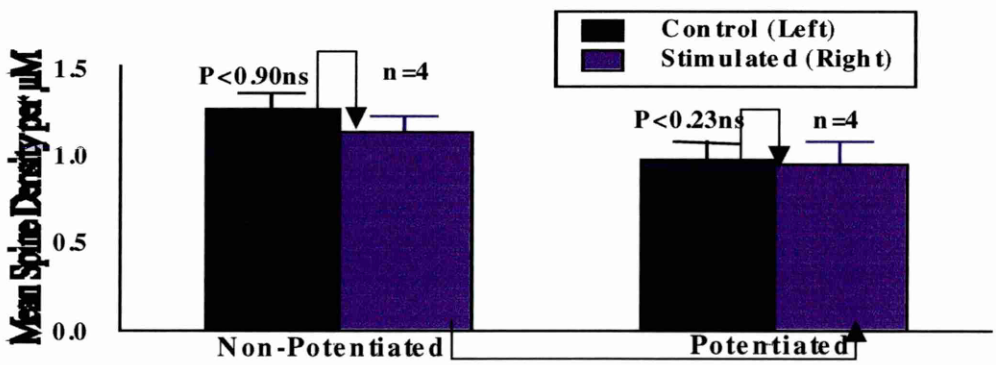
(i) Spine density changes in potentiated aged rats.

(ii) Spine density changes in non-potentiated aged rats.

The mean spine density in the control hemisphere of the potentiated animals was 0.969μm, and 0.952μm on the stimulated hemisphere. The mean spine density in the control hemisphere of the non-potentiated animals was 1.262μm and 1.132μm in the stimulated hemisphere.

One-way ANOVA analysis of the stimulated hemispheres from both potentiated and non-potentiated aged rats ($F_{1,7} = 2.084$, $P<0.27$) is not significant and student's t test within the animals indicates no significant change in spine density between hemispheres in the potentiated ($P<0.23$ ns), and non-potentiated rats ($P< 0.90$ ns). Whilst there is a difference in overall spine density (of left and right hemispheres combined) between non-potentiated and potentiated animals of approx 18%, this difference was not significant.

Figure 4.4: Mean Spine Density (± S.E) in the Middle Molecular Layer of Dentate Gyrus of Aged Rats 45 Minutes after High Frequency Stimulation.



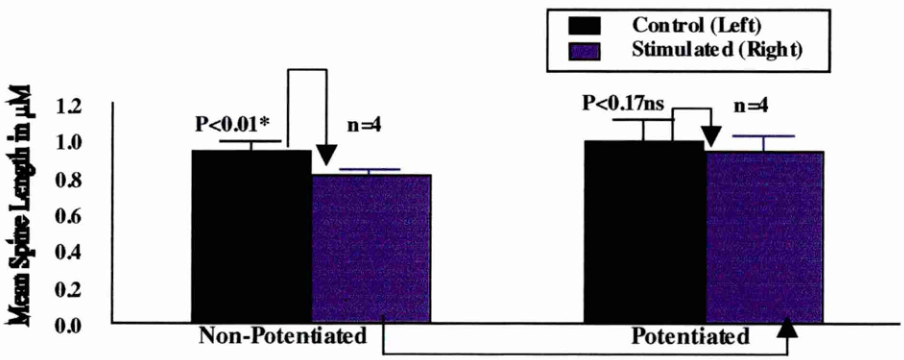
4.3.2 Spine Length: (Light Microscopy)

Modifications in spine length in the middle molecular layer of the dentate gyrus of aged rats were examined 45 minutes after LTP induction. The aged rats were divided into two categories as described above. The data are shown in Fig 4.5

(i) Spine length changes in potentiated aged rats.

(ii) Spine length changes in non-potentiated aged rats.

Figure 4.5: Mean Spine Length (± S.E) in the Middle Molecular Layer of Dentate Gyrus of Aged Rats (22months) 45 Minutes after High Frequency Stimulation of the Perforant Path.



The mean spine length in the potentiated animals, control hemisphere was 1.036µm, and 0.943µm on the stimulated hemisphere. The mean spine length in the non-potentiated animals, control hemisphere was 0.950µm, and 0.812µm on the stimulated hemisphere.

One way ANOVA analysis of the stimulated hemisphere with regards to spine length from both potentiated and non-potentiated aged rats ($F_{1,7}$=1.753, P<0.20) is not significant and student's t test within the group indicates a significantly shorter spine length in non-potentiated rats (P< 0.01*), but no significant change in potentiated rats (P< 0.17ns).

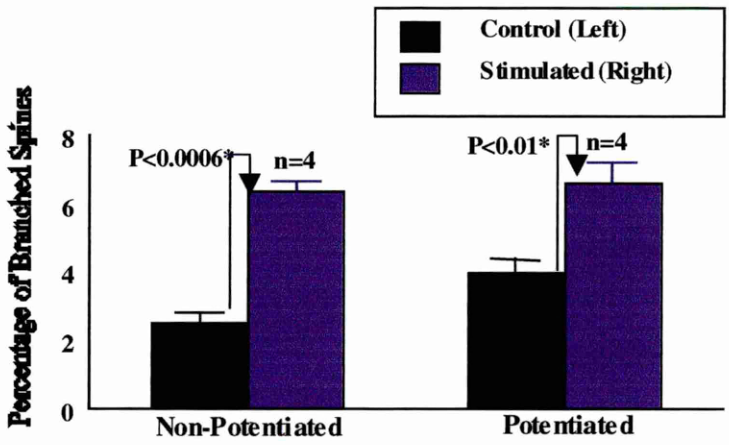
4.3.3 Percentage of Branched Spines: (Light Microscopy)

The percentage of branched spines in the middle molecular layer of the dentate gyrus of aged rats 45 minutes after LTP induction is shown in Fig 4.6. The aged rats were divided into the two categories as described in section 4.3.1:

(i) Percentage of branched spines in potentiated aged rats.

(ii) Percentage of branched spines in non-potentiated aged rats.

Figure 4.6: Percentage (± S.E) of Branched Spines in the Middle Molecular layer of Dentate Gyrus of Aged Rats 45 Minutes after High Frequency Stimulation.



The mean percentage of branched spines in the potentiated animals, control hemisphere was 3.94% and 6.57% in the stimulated hemisphere. The mean percentage of branched spines in the non-potentiated animals, control hemisphere was 2.53% and 6.37% in the stimulated hemisphere.

Student's t test indicates significant increases in the stimulated hemispheres of both non-potentiated rats ($P< 0.0006^*$) and potentiated rats ($P< 0.01^*$).

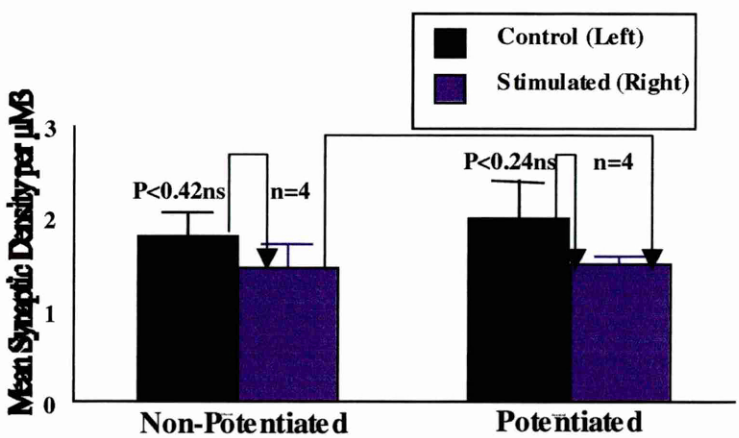
4.3.4 Numerical Synaptic Density: (Electron Microscopy)

The numerical synaptic densities of the synaptic types combined, whether on dendritic shafts or spines in the middle molecular layer of dentate gyrus of aged rats 45 minutes after LTP induction are shown in (Fig 4.7). The aged rats were divided into two groups (N = 4) each based on whether or not they had been potentiated following high frequency stimulation (Fig 4.3).

(i) Numerical synaptic density changes in potentiated aged rats.

(ii) Numerical synaptic density changes in non-potentiated aged rats.

Figure 4.7: Mean Numerical Synaptic Density (of all synapse types) in the Middle Molecular Layer of Dentate Gyrus of Aged Rats 45 Minutes after High Frequency Stimulation. Vertical bars represent standard errors (± S.E).



The mean synaptic density in the potentiated animals, control hemisphere was 2.01 per μm^3 and 1.497 per μm^3 in the stimulated hemisphere indicating a decrease of approx 25%. The mean synaptic density in the non-potentiated animals, control hemisphere was 1.79 per μm^3 and 1.46 per μm^3 on the stimulated hemisphere, a decrease of approx 18%.

One-way ANOVA analysis ($F_{1,7}$ =2.957, P<0.88) for the stimulated hemispheres from both potentiated and non-potentiated rats is not significant. Whilst this trend is towards a reduction in synaptic density in the stimulated hemisphere irrespective of the degree of potentiation. The data after student's t test within the group is also not statistically significant P<0.24 in the potentiated and P<0.42 in the non-potentiated rats.

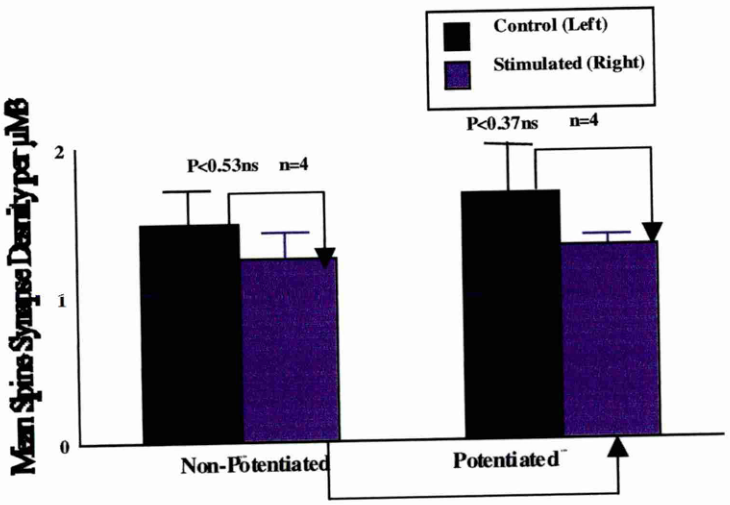
4.3.5 Numerical Density of Spine Synapses: (Electron Microscopy)

The numerical density of spine synapses in the middle molecular layer of dentate gyrus of aged rats 45 minutes after LTP induction are shown in (Fig 4.8). The aged rats are divided into two groups (N = 4) as described in section 4.3.1.

(i) Numerical density of spine synapses changes in potentiated aged rats.

(ii) Numerical density of spine synapses changes in non-potentiated aged rats.

Figure 4.8: Mean Density (± S.E) of Spine Synapses in the Middle Molecular Layer of Dentate Gyrus of Aged Rats 45 min after High Frequency Stimulation.



The mean spine synapse density in the potentiated animals, control hemisphere was 1.667 per μm^3 and 1.315 per μm^3 in the stimulated hemisphere. The mean spine synapse density in the non-potentiated animals, control hemisphere was1.48 per μm^3, and 1.24 per μm^3 on the stimulated hemisphere.

One-way ANOVA analysis for the stimulated hemispheres of both potentiated and non-potentiated aged rats ($F_{1,7} = 2.55$, $P < 0.69$) is not significant and student's t test within the group indicates no significant change in numerical density of spine synapses in the potentiated ($P<0.37$ ns) and non-potentiated ($P<0.53$ ns). However, the mean density of spine synapses decreases (approx 16-20%) in the stimulated hemisphere of potentiated and non-potentiated aged rats.

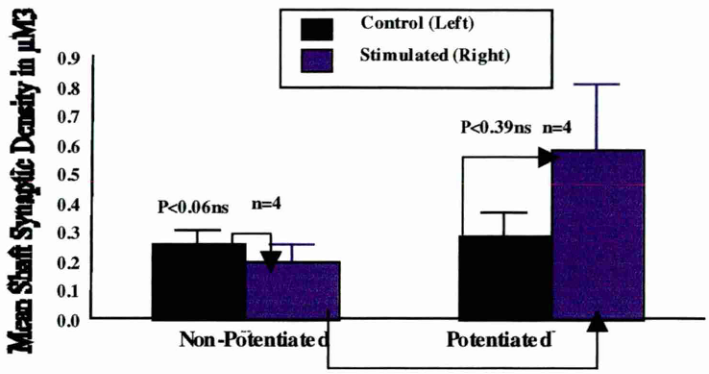
4.3.6 Numerical Density of Shaft Synapses: (Electron Microscopy)

The numerical density of shaft synapses in the middle molecular layer of dentate gyrus of aged rats 45 minutes after LTP induction are shown in (Fig 4.9). The aged rats were divided into two groups (N = 4) as described in section 4.3.1.

(i) Numerical density of shaft synapses changes in potentiated aged rats.

(ii) Numerical density of shaft synapses changes in non-potentiated aged rats.

Figure 4.9: Mean Density (± S.E) of Shaft Synapses in the Middle Molecular Layer of Dentate Gyrus of Aged Rats 45 Minutes after High Frequency Stimulation.



The mean density of shaft synapses in the potentiated animals, control hemisphere was 0.286 per μm^3, and 0.578 per μm^3 on the stimulated hemisphere. The mean density of shaft synapses in the non-potentiated animals, control hemisphere was 0.26 per μm^3, and 0.20 per μm^3 on the stimulated hemisphere.

One-way ANOVA analysis of the stimulated hemispheres of both potentiated and non-potentiated aged rats ($F_{1,7} = 0.778$, $P<0.15$) is not significant and student's t test within the group indicates no significant change in numerical density of shaft synapses in the potentiated ($P<0.39$ ns) and non-potentiated ($P<0.06$ ns). The mean density of shaft synapses increases in the stimulated hemisphere of potentiated aged rats.

4.3.7 Percentage of Perforated Synapses: (Electron Microscopy)

The percentage of perforated synapses (simple, complex & total) in the middle molecular layer of the dentate gyrus of aged rats 45 minutes after LTP induction. The aged rats were divided into two categories (N = 4) those which were potentiated and those which failed

to show potentiation as decribed in section 4.3.1. The data is shown in (Fig 4.10), an example of a complex perforated synapse is illustrated in (Fig 4.11).

(i) Percentage of simple perforated synapses in potentiated aged rats.

(ii) Percentage of complex perforated synapses in potentiated aged rats.

(iii) Percentage of total perforated synapses in potentiated aged rats.

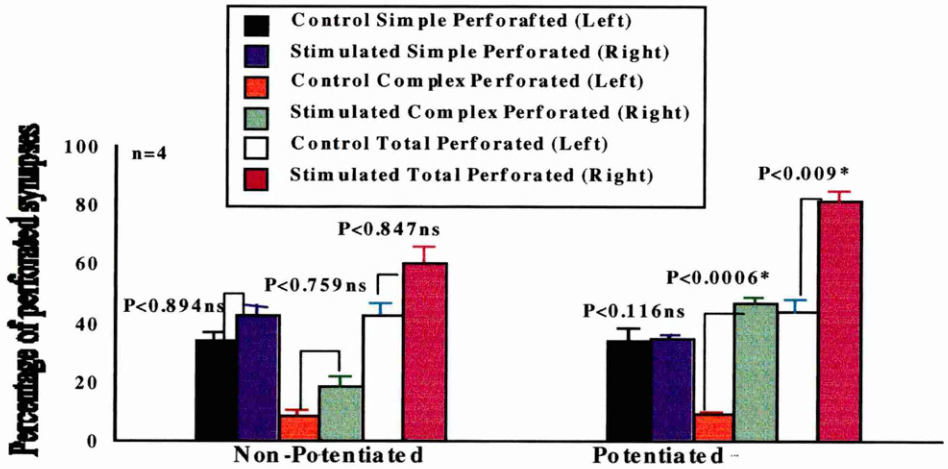
(iv) Percentage of simple perforated synapses in non-potentiated aged rats.

(v) Percentage of complex perforated synapses in non-potentiated aged rats.

(vi) Percentage of total perforated synapses in non-potentiated aged rats.

The definition of perforated synapses was given in the earlier section (1.8.5.3) and a diagrammatic classification of perforated synapses is shown also (Fig 1.9).

Figure 4.10: Mean Percentage (± S.E) of Perforated Synapses in the Middle Molecular Layer of Dentate Gyrus of Aged Rats 45 Minutes after High Frequency Stimulation.



Perforated Synapses in Potentiated Animals:

The mean percentage of perforated synapses in the potentiated animals for simple perforated synapses was 34.43% in the control hemisphere and 34.74% in the stimulated

hemisphere; for complex perforated synapses it was 9.46% in the control hemisphere and 46.76% in the stimulated hemisphere and for total perforated synapses 43.89% in the control hemisphere and 81.50% in the stimulated hemisphere.

Perforated Synapses in Non-Potentiated Animals:

The mean percentage of perforated synapses in the non-potentiated animals, simple perforated synapses was 33.78% in the control hemisphere and 42.61% in the stimulated hemisphere, for complex perforated synapses it was 8.84% in the control hemisphere and 18.11% in the stimulated hemisphere and for total perforated synapses 42.61% in the control hemisphere and 60.72% in the stimulated hemisphere).

The student's t test indicates in the potentiated animals there are no significant differences between stimulated and control hemispheres for simple perforated synapses ($P<0.16$), but there is an significant increase ($P<0.009$*) in total perforated synapses in the stimulated hemisphere and this is due to an significant increase ($P<0.0006$*) in the percentage of complex perforated synapses. Which increases by 3 times compared to the control hemisphere.

4.4 Discussion:

Dendritic Spine and Synaptic Densities:

These data show alterations in dendritic spines and synapses within the dentate molecular layer 45 minutes after high frequency stimulation in aged rats. The mean spine and synaptic densities show trends to be lower in the stimulated compared to the control hemispheres of potentiated rats, but the differences are not significant. These findings are in accord with previous findings by Desmond and Levy, (1986) who concluded that there is a trend for decrease in the number of synaptic contacts in the same area of the dentate gyrus 10min after LTP. More recently, a tendency towards decreased spine density was confirmed by (Rusakov, *et al.* 1997) 24 hrs after LTP induction. Meanwhile, in an electron microscopy study using 3D-reconstruction of dendritic spines from the dentate gyrus in two rats, an increase rather than a decrease in spine density was found 30 min after the induction of LTP (Trommald, *et al.*, 1990).

Spine and Shaft Synaptic Density:

No significant changes in numerical density of either shaft and spine synapses were observed, but the mean numerical density of shaft synapses was (~ 35%) higher in the potentiated animals, in agreement with earlier findings by Lee, *et al.*, (1980) after LTP in the CA1 region of the hippocampus.

When these results are compared with those from other laboratories, several factors should be considered, including the sampling and measurement strategies, the time post-tetanus analyzed, and the experimental design. These differences cannot be explained by inadequate silver impregnation with the Golgi method: in spite of the fact that there is no

technique for directly quantifying how well the dendrites are impregnated with silver, the visible spine densities (uncorrected for hidden spines) estimated here ($0.952\mu m^{-1}$ for potentiated; $1.132\mu m^{-1}$ for non-potentiated aged rats, (Fig 4.4) are in fact in good correspondence with the longitudinal spine density ($1.01\mu m^{-1}$) recently reported by (Trommald and Hulleberg, 1997) using serial section electron microscopy technique.

Many other studies have reported no changes in total synaptic spine contacts after LTP in the Schaffer collateral/ commisural synapse in CA1 (Lee, *et al.*, 1980; Chang and Greenough, 1984; Gomez, *et al.*, 1990; Schuster, *et al.*, 1990; Chang, *et al.*, 1991; Geinisman, *et al.*, 1991; Hosokawa, *et al.*, 1995; Buchs and Muller, 1996; Sorra and Harris, 1998).

Spine Length:

The mean spine length ($0.943\mu m^{-1}$) was higher in the stimulated hemisphere of potentiated animals than in stimulated but non-potentiated animals ($0.812\mu m^{-1}$). Whilst this difference was not statistically significant, the difference between spine length of the control & stimulated hemisphere of non-potentiated rats was significant with a reduction in mean spine length of ~14%. An increase in spine length of about $0.8\mu m^{-1}$ within 30 minutes of LTP induction was reported by (Trommald, *et al.*, 1990) in the same area of the dentate gyrus. Real-time dendritic spine studies in area CA1 of living hippocampal slices using confocal microscopy (Hosokawa, *et al.*, 1995) reported longer spines to be a common feature after chemical induction of LTP. Similarly (Papa and Segal, 1996) observed elongation of spines after treating cultured hippocampal neurons for 24 hrs with tetrodotoxin (TTX). The most dominant dendritic spines are long, thin stalks and small

bulbous ending comprising 72% of the total percentage of spines (Peters and Kaiserman-Abramof, 1970) and studies in normal aging human brain suggest an increase in mean length of the postsynaptic contact zone (Adams, 1987). The most likely explanation from a biophysical point regarding the trend to longer dendritic spines in aged rats, which sustained LTP, is that they may be decreasing the spine neck resistance and thereby increasing the amplitude of the EPSP, so facilitating synaptic efficacy.

Branched Spines:

The most notable change is a highly significant increase in the percentage of branched spines, but this occurs irrespective of whether the animals are potentiated or not suggesting a direct effect of high frequency stimulation on spine morphology *per se*. This is in conformity with the observations of Trommald, *et al.*, (1990) that bifurcating spine density increased roughly three times 30 minutes after LTP induction, although she did not report on spine branching in stimulated but non-potentiated tissue. Rusakov, *et al.*, (1997) observed a reduction in spine density 24 hours after LTP induction, possibly associated with retraction and fusion with branching. It is important to emphasize below the significant benefits of the Golgi impregnation technique, since it permits one to recognize and examine, in each animal, numerous dendritic segments and accordingly, thousands of dendritic spines related to distinct cells from the population of interest.

The findings suggests that in aged rats LTP is sustained better in those animals with fewer but longer dendritic spines, but potentiation does not cause significant morphological changes of spines *per se* at the short time examined after tetanic stimulation. Spine branching is caused by the process of high frequency stimulation and is not a result of the occurrence of LTP.

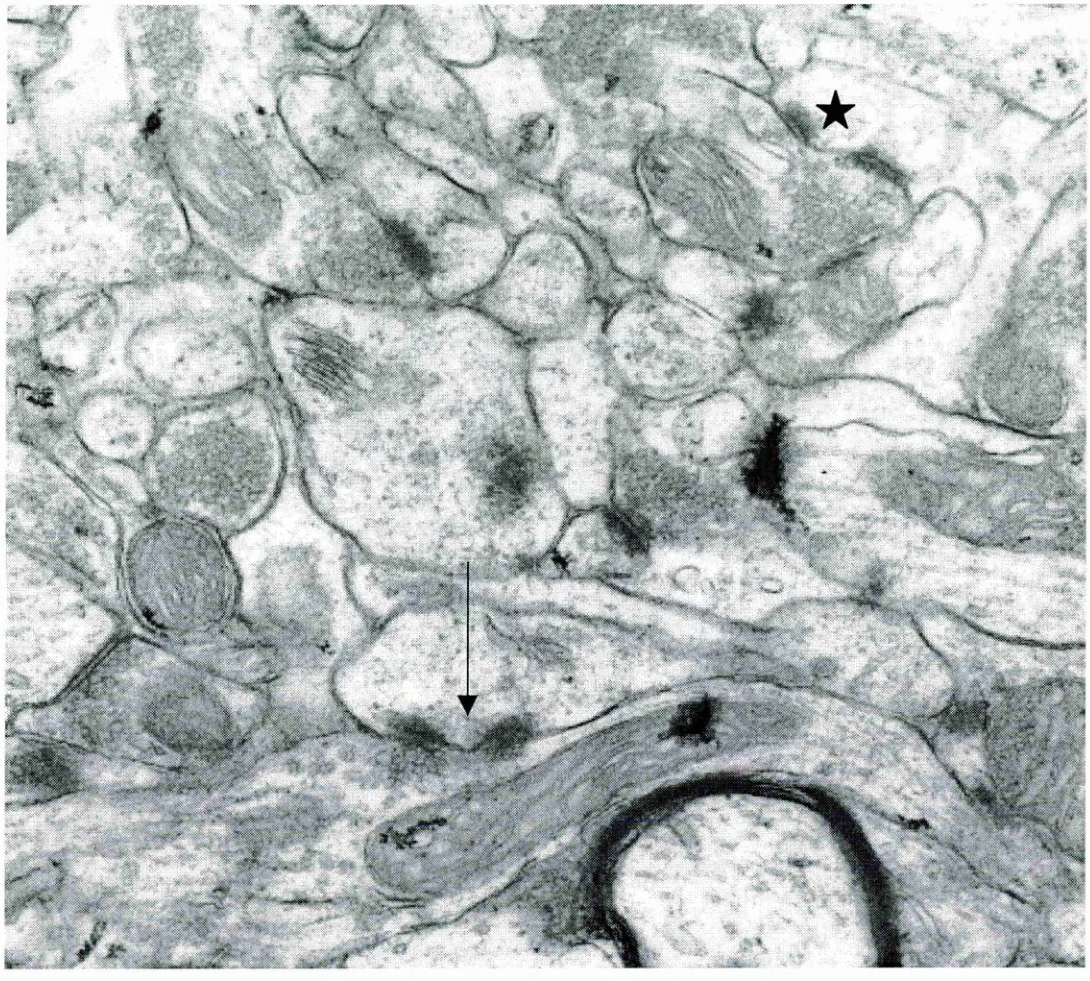
Perforated synapses:

The most notable synaptic changes occurred in relation to synaptic perforations. A significant increase occurred in the percentage of perforated synapses (complex and total) in the stimulated hemisphere of potentiated rats 45 minues after LTP induction, but not in the non-potentiated animals (Fig 4.10). Geinisman, *et al.*, (1991, 1992b, 1994) also observed a significant increase in multiple complex perforated synapses in aged rats after stimulating then (fifteen 20-ms burst of 400 Hz) on each of four consecutive days and sacrificed 1 hours after the fourth stimulation and (Geinisman, *et al.*,1992c; deToledo-Morrell, *et al.*,1988) reported an increase in the number of double headed spines and perforated synapses, following kindling.

There is a considerable variation in the ability of aged animals to sustain long-term potentiation (LTP) following induction. This variation enabled us with the opportunity to determine what types of synaptic alterations were dependent upon the ability to sustain potentiation. Several groups (Barnes, 1979; Lynch, 1998; deToeldo-Morrell and Morrell, 1985) have reported a deficit in LTP of aged rats. Age-related decrease in membrane fluidity arises from increases in membrane concentrations of cholestrol (Zhang, *et al.*, 1996), coupled with a decrease in membrane arachidonic acid (Murray and Lynch, 1998). Several groups have indicated that the later phases of LTP require protein synthesis, which is essential for morphological changes. It has also been shown recently that aged rats fed on a diet supplemented with arachidonic acid for 8 weeks sustained LTP better than aged rats fed on control diet (McGahon, *et al.*, 1997). The data described above suggests that the impaired in the aged rats to sustain LTP results from several age-related changes in the hippocampus. Several groups have also observed age-related decrease in

NMDA receptor function. Recently it was reported that the percentage of concave perforated synapses increased by ~20% in middle molecular layer of the LTP animals compared to the control animals (Weeks, *et al.*, 1999). The possible physiological relevance lies in the individual variations of aged rats to convert a simple perforated synapse into a complex perforated synapse in order sustaining LTP.

Figure 4.11: Perforated synapses on dendritic shaft (Axodendritic Synapse) (arrow) and on spines (Axospinous Synapse) (star).



Chapter 5

Plasticity of Dendritic Spine Morphology and synaptic density, and the Ability of Young Rats to Sustain LTP in the Dentate Gyrus, 45 Min after Tetanic Stimulation of the Perforant Path

5.1 Introduction:

Plasticity is defined as the ability of synaptic contacts to undergo rapid changes in their structure following repetitive utilization, and this synaptic structural plasticity underlines the basis of learning and memory. It has been proposed that dendritic spines constitute the main focal point of long-term synaptic alterations associated with functional plasticity of neurons (Peters, *et al.*, 1976). At the ultrastructural level extensive analyses of hippocampal neurons has indicated that dendritic spines undergo significant morphological changes following exposure to various stimuli, such as kindling (Morrell, *et al.*, 1986), tetanic stimulation (Desmond and Levy, 1988), passive avoidance (O'Malley, *et al.*, 1998) or even natural (Moser, *et al.*, 1994) or hormonal (Woolley, *et al.*, 1990) stimulation. As apposed to aged animals, the majority of young rats generally tend to potentiate upon stimulation. Earlier studies using single and serial section analysis (Desmond and Levy, 1986; Lynch, *et al.*, 1988; Geinisman, *et al.*, 1992b) found that LTP induction caused significant increases in synaptic and spine parameters in the dentate gyrus at various times after stimulation, and using different levels of high frequency stimulation.

The aim of this part of the study was to use the same unbiased methods and similar high frequency stimulation in young rats, to examine the morphology of spines and synapses after LTP, and to compare the data with aged animals.

5.2 Methods:

Methods, perfusion, fixation and procedure for light and electron microscopy are described in detail in chapter 3, while estimation of thickness of ultrathin sections, and the unbiased methods for estimation of spine and synaptic parameters were given in

chapter 4. Inorder to ensure that the research was conducted blind, the animals were coded at Trinity College, Dublin and the code was not released until all the data was collected, enetered, and ready for statistical analysis.

5.3.1 The Density of Spines and Synapses:

From each hemisphere, 15-20 images of dendritic fragments in the middle molecular layer of the dentate gyrus were captured from Golgi stained hippocampal sections. Spine density was estimated using 'tilting disector technique' and ~ 24 'disector' images were acquired from each pair of serial ultrathin sections, of the left, and similar numbers from the right hemisphere. As in chapter 4 quantification of synaptic density Nv syn per μm^3 was based on (Sterio, 1984) for all visible synapses, without distinction between types, i.e. whether with symmetric or asymmetric synaptic junctions, or irrespective of their location on dendritic spines, or shafts. Since, asymmetrical synapses comprised 90% of the total visible synapses further classification concerned only asymmetrical synapses and whether they occurred on dendritic spines or on shafts.

5.3.2. Data analysis and statistical design:

Inter-hemispheric analysis of spine density, spine length, total synaptic density, density of spine synapses, density of shaft synapses, percentage of bifurcating spines and perforated synapses (simple and complex) was performed using student 't' test where applicable. Differences were considered significant at $P<0.05$. The following factors were examined:

a) Hemispheric (left/ right) differences, and

b) The effect of high frequency stimulation on young rats

5.3.3 Synaptic plasticity changes in young rats 45 minutes after LTP induction:

Long-term potentiation (LTP) was elicited unilaterally in young (3-month-old) male rats in the right hemisphere (as in aged animals). A total of N= 6 young male rats were stimulated. All six young rats sustained LTP 45 minutes after induction (see Fig 4.3). Each animal serves as its own control, because the EC-DG system is largely unilateral (Steward and Vinsant, 1983). Thus the non-tetanized hemisphere (left) was taken as the control.

5.4 Results:

5.4.1 Numerical Spine Density: (Light Microscopy)

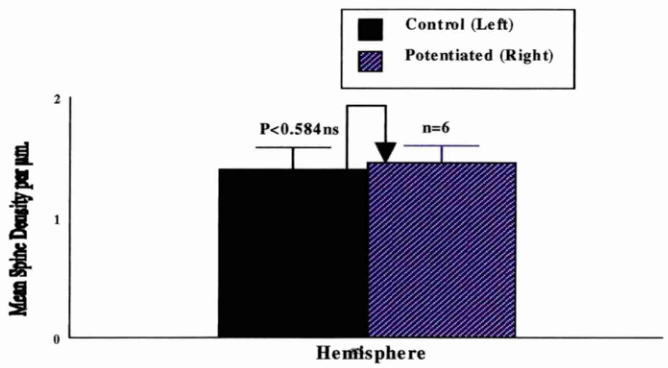
The alterations in spine density in the middle molecular layer of the dentate gyrus of young rats 45 minutes after LTP induction are shown in Fig 5.1:

(i) Spine density in the control hemisphere

(iii) Spine density in the stimulated hemisphere.

The mean spine density in the control hemisphere was 1.40μm, and 1.46μm in the stimulated hemisphere. Student's t test analysis indicates no significant change in spine density between the potentiated hemisphere and (contralateral) control hemisphere ($P<0.584$ ns).

Figure 5.1: Mean Spine Density in the Middle Molecular Layer of Dentate Gyrus of Young Rats 45 Minutes after High Frequency Stimulation. Vertical bars represents standard errors (± S.E).



5.4.2 Spine Length: (Light Microscopy)

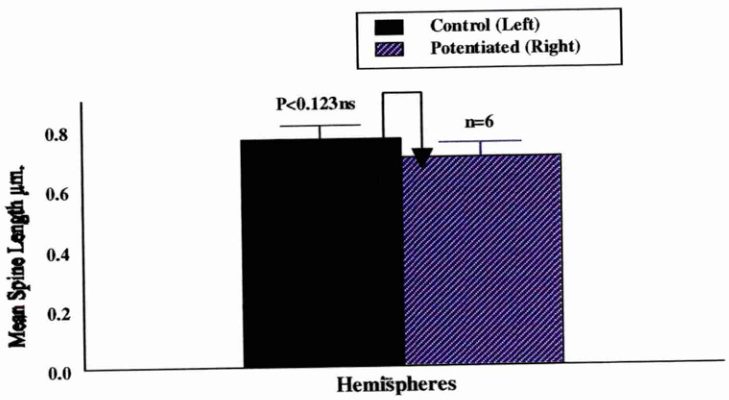
The changes in spine length in the middle molecular layer of the dentate gyrus of young rats 45 minutes after LTP induction are shown in Fig 5.2.

(i) Spine length in the control hemisphere.

(ii) Spine length in the stimulated hemisphere.

The mean spine length in the control hemisphere was 0.767μm and 0.700μm in the stimulated hemisphere. Student's t test indicates no significant change in spine length in the potentiated hemisphere compared to the (contralateral) control hemisphere (P<0.123 ns).

Figure 5.2: Mean Spine Length (± S.E) in the Middle Molecular Layer of Dentate Gyrus of Young Rats 45 Minutes after High Frequency Stimulation.



5.4.3 Percentage of Branched Spines: (Light Microscopy)

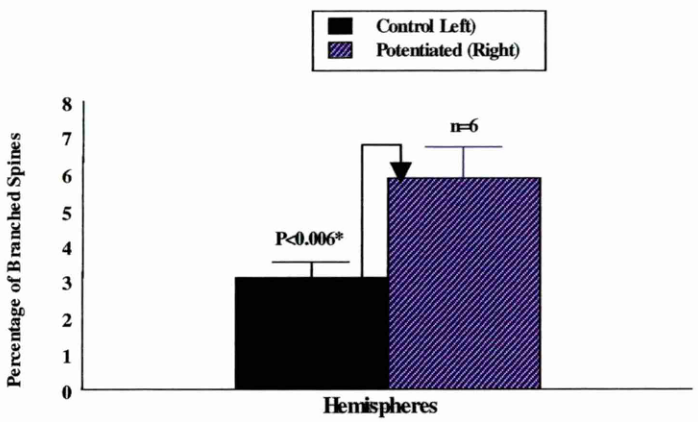
The changes in percentage of branched spines in the middle molecular layer of the dentate gyrus of young rats 45 minutes after LTP induction are shown in Fig 5.3.

(i) Percentage of branched spines in the control hemisphere.

(ii) Percentage of branched spines in the stimulated hemisphere.

The percentage of branched spines in the control hemisphere was 3.09%, and 5.89% in the stimulated hemisphere. Student's t test indicates a significant change in the percentage of branched spines in the potentiated hemisphere comapred to the (contralateral) control hemisphere (P<0.006*).

Figure 5.3: Mean percentage (± S.E) of branched spines in the middle molecular layer of the dentate gyrus of young rats 45 minutes after High Frequency Stimulation.



5.4.4 Numerical Synaptic Density: (Electron Microscopy)

The numerical synaptic density of the synaptic types combined, whether on dendritic shafts or spines, in the middle molecular layer of dentate gyrus of young rats 45 minutes after LTP induction is shown in (Fig 5.4).

(i) Numerical synaptic density in the control hemisphere.

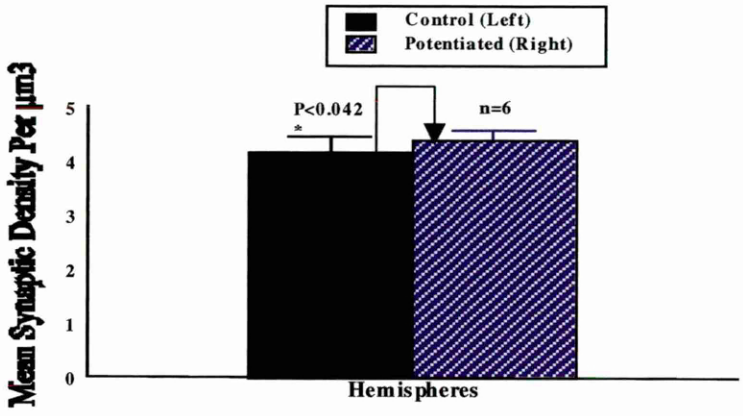
(ii) Numerical synaptic density in the stimulated hemisphere.

The mean synaptic density in the young animals was 4.16 per μm^3 in the control hemisphere and 4.42 per μm^3 in the stimulated hemisphere. Student's t test indicates a

significant increase (~7%) in total synaptic density in the stimulated hemisphere

(P<0.042*).

Figure 5.4: Mean Numerical Synaptic Density (of all synapse types) in the Middle Molecular Layer of Dentate Gyrus of Young Rats 45 Minutes after High Frequency Stimulation. Vertical bars represent standard errors (± S.E).



5.4.5 Numerical Density of Spine Synapses: (Electron Microscopy)

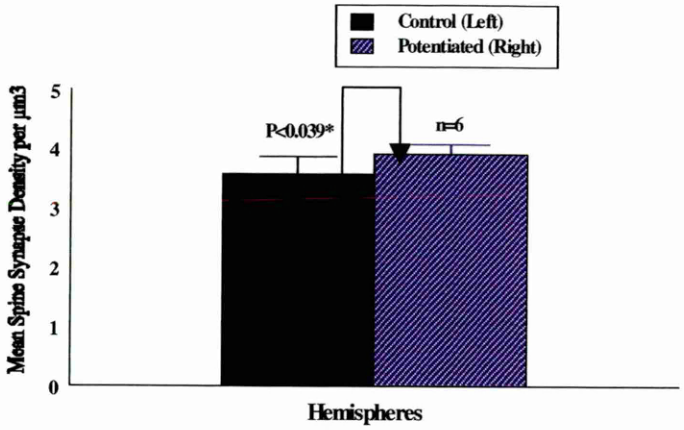
The numerical density of spine synapses in the middle molecular layer of the dentate gyrus of young rats 45 minutes after LTP induction is shown in (Fig 5.5).

(i) Numerical density of spine synapses in the control hemisphere.

(ii) Numerical density of spine synapses in the stimulated hemisphere.

The mean spine synapse density in the young animals was 3.57 per μm^3 in the control hemisphere and 3.90 per μm^3 in the stimulated hemisphere, an increase of approximately 10%. Student's t test indicate that the increase in numerical density of spine synapses in the stimulated hemisphere is statistically significant (P<0.039*).

Figure 5.5: Mean Density (± S.E) of Spine Synapses in the Middle Molecular Layer of Dentate Gyrus of Young Rats 45 Minutes after High Frequency Stimulation.



5.4.6 Numerical Density of Shaft Synapses: (Electron Microscopy)

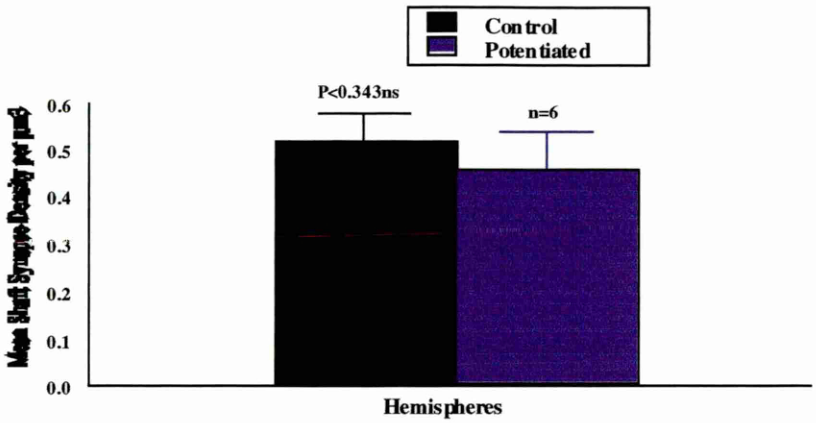
The numerical density of shaft synapses in the middle molecular layer of dentate gyrus of young rats 45 minutes after LTP induction is shown in (Fig 5.6).

(i) Numerical density of shaft synapses in the control hemisphere.

(ii) Numerical density of shaft synapses in the stimulated hemisphere.

The mean density of shaft synapses in the young animals was 0.512 per μm^3 in the control hemisphere and 0.450 per μm^3 in the stimulated hemisphere, a decrease of 12%. Student's t test indicates no significant change in the numerical density of shaft synapses in the stimulated hemisphere compared to the (contralateral) control hemisphere (P< 0.343ns).

Figure 5.6: Mean Density (± S.E) of Shaft Synapses in the Middle Molecular Layer of Dentate Gyrus of Young Rats 45 Minutes after High Frequency Stimulation.



5.4.7 Percentage of Perforated Synapses: (Electron Microscopy)

The percentage of perforated synapses (simple, complex & total) in the middle molecular layer of the dentate gyrus of young rats 45 minutes after LTP induction is shown in (Fig 5.7).

(i) Percentage of simple perforated synapses in the control hemisphere.

(ii) Percentage of complex perforated synapses in the control hemisphere.

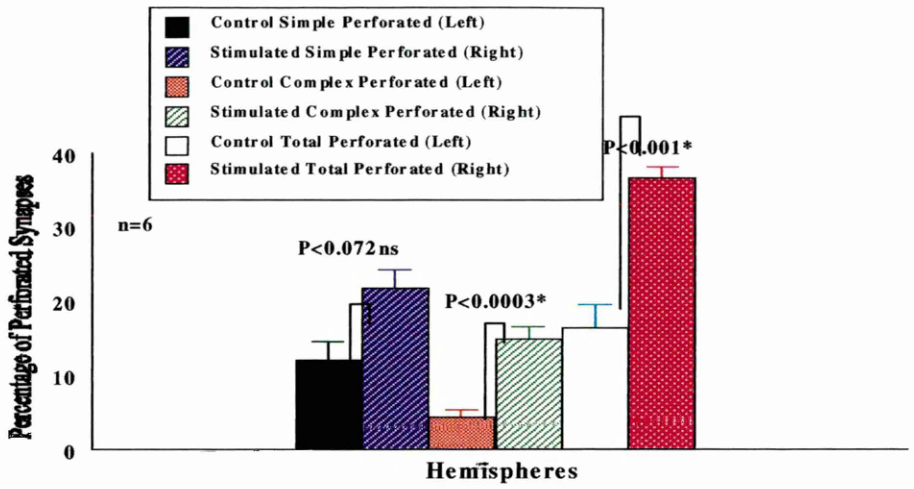
(iii) Percentage of total perforated synapses in the control hemisphere.

(iv) Percentage of simple perforated synapses in the stimulated hemisphere.

(v) Percentage of complex perforated synapses in the stimulated hemisphere.

(vi) Percentage of total perforated synapses in the stimulated hemisphere.

Figure 5.7: Mean Percentage (± S.E) of Perforated Synapses in the Middle Molecular Layer of Dentate Gyrus of Young Rats 45 Minutes after High Frequency Stimulation.



The mean percentage of simple perforated synapses in young rats was 11.93% in the control hemisphere and 21.83% in the stimulated hemisphere. For complex perforated synapses the values were 4.42% in control hemisphere and 14.82% in the stimulated hemisphere and for total perforated synapses 16.35% in the control hemisphere and 36.70% in the stimulated hemisphere. Student's t test indicates no significant changes between stimulated & control hemisphere in simple perforated synapses (P<0.072ns) although it shows a trend towards an increase (approx. 9%). However, a significant increase occurred (~10%) in complex perforated synapses (P<0.0003*) and thus the total perforated synapses increases (~19%) in the stimulated hemisphere of young rats (P<0.0018*).

5.5 Discussion:

The data here provides evidence of anatomical alterations in both dendritic spines and synapses within the dentate molecular layer after LTP induction in young (3 month old) rats, when examined at the light and electron microscope level. However there are no significant changes in spine density or spine length 45 minutes following induction of LTP. Thus the present observation (Fig: 5.1) showing a trend towards increased spine density lends support to the earlier findings that spine density increases during development (Malley, *et al.*, 1998; Eckenhoff and Rakic, 1991; Harris, *et al.*, 1989; Papa, *et al.*, 1995; Papa and Segal, 1996). Conversely, our observation of shrinkage of spines ($0.767\mu m^{-1}$ in the control hemisphere and $0.700\mu m^{-1}$ in the stimulated hemisphere) partly confirms previous studies showing that excessive stimulation causes elimination of spines (Muller, *et al.*, 1993; Papa and Segal, 1996) in hippocampal slices after chronic epileptic seizures and synaptic activity (Fig: 5.2).

The significant finding of the experimental study in the hippocampal dentate gyrus of young rats is the marked and significant increase in the percentage of bifurcating spines (Fig: 5.3) and the density of axospinous synapses that are distinguished as complex perforated (Fig 5.7). There is ~ 10-15% increase in these class of perforated synapses only 45 minutes after LTP induction. These structural changes are highly specific since they involve only one particular synaptic subtype. Moreover, it occurs only in the terminal synaptic field of the stimulated middle molecular layer, not the inner. Thus, these results are in conformation with previous findings by Nieto-Sampedro, *et al.*, (1982), who suggested that naïve 3 month young rats showed an increase in complex

perforated synapses by 3.7% in the control hemisphere, while Geinisman, *et al.*, (1986, 1987, 1988, 1989) reported that complex multiple perforated synapses increased by 19.7% and 12.8% in 5 month old rats after LTP induction and kindling respectively. The present data is also in agreement with and confirms earlier report by Buchs and Muller, (1996) that 60% of the labeled synapses had perforated postsynaptic densities in hippocampal CA1 region 30-40 minutes after LTP induction. The increase in spine branching or bifurcating spines is also highly significant but given that this particular parameter changes similarly in stimulated but non-potentiated aged rats, it is possible that electrical stimulation *per se* plays a role in spine branching. What marks the present data from previous studies on synaptic changes after LTP is the observation of a significant increase in the overall synaptic density, in addition to that, the increased complex perforated synapses found by Geinisman, *et al.*, (1988; 1989; 1995). There are several reasons why there could be differences with Geinisman and other investigators. Firstly, as opposed to Geinisman's study carried out 3 days after initial potentiation, the present study was carried out only 45 minutes after stimulation. Secondly, in contrast to many earlier studies (Geinisman, *et al.*, 1976; Lee, *et al.*, 1980), the present investigation ultilized unbiased methods.

Together, with other recent studies (Bundman, *et al.*, 1994a,b; Moser, *et al.*, 1994; Malley, *et al.*, 1998), the present results indicate that the dendritic spine is a dynamic structure, extremely responsive to alterations in circulating conditions. It has been suggested, that spines play an important role in the regulation of postsynaptic [Ca^{2+}] (Guthrie, *et al.*, 1991; Koch and Zador, 1993; Wickens, 1988). The data indicates that young rats are able to sustain LTP induction due to their ability to form complex

perforated synapses, increase in branched spines and an overall increase in spine and synaptic density compared to the aged rats. Therefore, three processes could be taking place simultaneously during this phase CNS development: (i) shortening of existing spines due to the tetanic stimulation, probably facilitating the rise in dendritic [Ca^{2+}], (ii) formation of complex perforated synapses and (iii) formation of new spines and branched spines.

Finally, serial section analyses from EM and light microscopy studies from Golgi impregnation have revealed gross changes in the total spine and synapse densities during development, but serial EM is required to delineate unambiguously any changes in the frequency of spines and synapses with different geometries (Harris, *et al.*, 1992). The present experimental findings further substantiate the earlier statement by Turner, *et al.*, (1985) that Golgi-stained preparations appear to reflect accurately electron microscopic estimates.

General Discussion

This thesis has examined anatomical plasticity following long-term potentiation in young and old animals. The basic mechanism of neuronal modelling caused by environment may be through the process of synapse turnover. The relationship between synapse turnover and behaviour is still relatively unknown, but the logical assumption would be the ability of the brain to modify its synaptic circuitry in response to stimuli is someway related to the adaptive ability of the nervous system (Zola-Morgan and Squire, 1990). The process of learning could hasten or slow the process and thereby adapt to neuronal connectivity. (Lynch, 1986; Barnes, 1995; Ziv and Smith, 1996; Fischer, *et al.*, 1998).

Ageing mammals are generally associated with a decline in cognitive function, particularly for newly acquired information (Barnes, 1994). Age-related decline in memory dysfunction could be related to concomitant decline and alterations in synaptic plasticity of the hippocampal formation (Barnes, 1980, 1983; Thompson, *et al.*1994). A of recent research work using animal models for human amnesia support the role of hippocampal formation in age-related decline in memory function and synaptic plasticity (Mishkin, *et al.*, 1982; Zola-Morgan, 1984; 1986).

Comparisons with previous studies:

The role of dendritic spines and synapses in LTP has been extensively investigated (Fifkova, *et al.* 1981; Harris, *et al.*, 1992; Sorra, *et al.*, 1998; Rusakov, *et al.*, 1997; Geinisman, *et al.* 1994, 1995). The present work on aged rats (potentiated and non-potentiated) as discussed in chapter 4 has shown that alterations in synaptic efficacy could play a role in the induction of LTP (Stewart, *et al.*1997; Dhanrajan, *et al.*1997,

1998). The data obtained via unbiased morphometric techniques at light and electron microscope level indicate a transient decrease in spine and synaptic densities in the stimulated hemisphere compared to the contralateral control hemisphere. These finding support the earlier work by Geinisman, *et al.*, (1976,1979) which showed that in naïve aged rats there is a decrease of approximately 30% in synapse density in the middle molecular layer of the dentate gyrus. Leuba (1983) recorded a 50% decrease in dendritic spines between the ages of six months and two years in mice. Desmond and Levy (1985, 1986) and Trommald (1990) reported in adult rats using rapid Golgi stain and serial EM reconstruction a corrected average spine density of 1.3 spines/μm and 1.2 spines/μm in the dentate gyrus respectively. Geinisman, *et al.*, (1987) reported in the fascia dentate a 17% decrease in the number of all axospinous synapses per neuron in memory-impaired old animals. The mean density of "shaft" synapses was higher in the potentiated rats, while the mean density of "shaft" synapses was lower in the non-potentiated rats. A similar increase in the incidence of "shaft" synapses (33%) was observed in the hippocampal CA1 pyramidal cells after LTP induction by Lee, *et al.*, (1980). The above increase in shaft synapses could be accounted for by a "retraction" of dendritic spines into the shaft synapses due to ageing. Therefore, the ability of an aged rat to hold LTP may depend on the presence of a higher density of "shaft" synapses.

Peters and Kaiser-Abramof, (1970) observed that 72% of the dendritic spines were long, thin stalk and had small bulbous endings. The mean spine length in the stimulated hemisphere of animals that sustained LTP was $0.943\mu m^{-1}$ and $0.812\mu m^{-1}$ in the animals that did not sustained LTP. Trommald, (1990) also reported an increase in spine length of

($0.8\mu m^{-1}$) in adult rats (n=2) 30 minutes after LTP induction in longitudinal dendritic fragment using 3D electron microscope reconstruction. Besides real time dendritic spine studies in CA1 region of living hippocampal slices using confocal microscopy Hosokawa, *et al.*, (1995) also reported longer spines to be a common feature after chemically induced LTP. The possible importance of spine length in the induction of LTP was suggested by Adams, (1986) who reported that the loss of synapses in the precentral gyrus of elderly human individuals could act as a stimulus to existing synaptic ultrastructures to increase the postsynaptic contact length of the remaining synapses. Physiological experiments suggest that synaptic transmission is enhanced in long and thin spines (Harris and Teyler, 1983, 1984). As the constricted spine neck would *enhance* synaptic transmission by amplying the potential generated in the spine heads via voltage–dependent calcium channels (Woolf, *et al.*, 1991).

Considerable evidence has accumulated that suggests the possible role of branched spines and perforated synapses in synaptic plasticity accompanying learning and memory (Geinisman, *et al.*, 1991a, 1995; deToledo-Morrell, *et al.*, 1984a, 1988b). As these synaptic junctions are presumed to be more efficacous in augmenting synaptic efficacy, they are regarded as a basic mechanism involved in learning and memory (Calverley and Jones, 1990; Wallace, 1991). In our study on aged rats, discussed in chapter 4, we exploited the natural variability among aged animals with respect to LTP induction. The complement of complex perforated synapses and density of total perforated synapses was significantly increased in the potentiated compared to the non-potentiated animals. Geinisman, *et al.*, (1994) also observed a significant increase in multiple, completely

partitioned transmission zones in aged rats that sustained LTP. It has been reported that memory deficient aged rats showed 22% fewer perforated synapses per neuron than in "memory intact" aged rats after spatial learning task Geinisman, *et al.*, (1986). Similarily, in aged rats that didn't sustain LTP, a decrease in complex perforated synapses and an increase in percentage of simple perforated synapses was observed. Here, the percentage of branched spines was significantly (2-3 times) higher in the aged rats after LTP induction (Dhanrajan, *et al.*, 1998). Similarly, Trommald, (1990) also observed a 2-3 times increase in branched spines density 30 minutes after LTP induction. These structural alterations involve only certain synaptic subtypes. In the case of aged rats the ability to sustain LTP depends on longer and thinner spines, formation of branched spines and specifically complex perforated synapses from simple perforated synapses. Therefore, the present results after synaptic potentiation may reflect interconversion of simple perforated synapses to complex perforated synapses and shaft synapses could represent structural intermediates in synaptic plasticity associated with LTP. Parsimony would favour the interconversion of dendritic spines and synapses, rather than complete remodelling of synapses involving elimination and addition of spines and synapses.

In order to understand the morphological substrate of learning and memory, young (3 month) old rats were stimulated for 45 minutes as discussed in chapter 5. Here we detected a small non-significant increase in spine density, but a significant increase in synaptic density. The present findings agree with earlier electron microscope reports where electrical stimulation of afferent fibres to the dentate gyrus was followed by enhanced spine and synaptic density Fifkova and Van Harreveld, (1977); Fifkova and Anderson, (1981) and Trommald, (1990). Recently O' Malley, *et al.*, (1998) has shown a

transient increase in hippocampal dentate spine density 6 hours after passive avoidance learning in young adult rats. However, despite claims to the contrary, no actual measurement of synaptic density was made by Trommald, (1990). A similar increase in spine density was observed in hippocampal CA1 cells following spatial learning (Moser, *et al.*, 1994). The present findings deviate from the findings by Desmond and Levy, 1983, 1986) who concluded no overall change in spine density, while Rusakov, *et al.*, (1997) concluded a decrease in spine density in the same region 24 hrs after LTP. We have no simple explanation for the deviation of our results from Desmond & Levy and Rusakov except for the time between the induction of LTP and perfusion of the animals. In the experimental findings of Rusakov, *et al.*, (1997) the animals were perfused 24 hrs after LTP induction compared to 45 minutes here. The reorganization of spine and synapses are known to occur within 2-10 minutes following induction (Desmond and Levy, 1986), so the experiment may have been closer to this initial reorganization time, after which spines are selectively lost.

The mean spine length was shorter in the stimulated hemisphere, while Trommald, (1990) and Papa and Segal (1996) reported an increase in spine length in adult rats and cultured hippocampal neurons respectively. In culture conditions dendritic spine length (1-1.5μm) were observed to proceed towards a glutamate gradient with a speed of up to a micron per 20 seconds (Boyer, *et al.*, 1998). Thus, a possible explanation for a shorter spine length could be the age of the animals, or the high frequency stimulation could be inducing "spine retraction" in developing young animals.

Trommald, (1990) and Fifkova and Van Harreveld, (1977) found an increase in the area of dendritic spines in the LTP material, which supports the significant increase in the density of "spine" synapses, while the density of "shaft" synapses decreased. Chang and Greenough, (1984) reported an increase in sessile shaft and spine synapses after LTP in the CA1 region, and also confirmed that the postsynaptic density length in spine synapseswas unchanged. As discussed earlier in section 5.5 shaft synapses could consititute an intermediate form capable of transforming into asymmetrical "spine" synapses as dendritic "shaft" synapses continue to be formed during development.

The major findings of the present study is that the induction of LTP in the dentate gyrus of young rats is followed by a marked and significant increase (i) in the percentage of bifurcating or branched spines, (ii) percentage of complex perforated and (iii) density of total perforated synapses. The present results complement the findings of Trommald, (1990) who observed a 2-3 times increase in bifuracting spines in adult rats (n=2) 30 minutes after LTP induction using 3D electron microscope reconstruction. Geinisman, *et al.*, (1993), Buchs & Muller (1996) and Desmond & Levy (1983, 1990) reported a 32% increase in perforated synapses after LTP induction.

Therefore, the increase in spine and synaptic density, shorter spine length, increased "spine" synapses, decreased "shaft" synapses, increased branched spines and perforated synapses observed after LTP induction could be due to the parallel mechanisms of "interconversion" and "synaptogenesis" theory (Steward and Vinsant, 1983). Nevertheless, the "synaptogenesis" theory (de novo synthesis after LTP) cannot be ruled out in the present experiment due to the age (3 months) of the rats.

Finally, this search for the morphological substrate for neural plasticity ended in a comparison of the physiological and morphological basis for the induction of LTP in the young and aged rats. Analysis of electrophysiological data indicates that young and old rats under comparison did not differ significantly in terms of the extent of potentiation observed (Fig 4.3). However, almost 50% of the aged rats failed to sustain LTP at 45 min. Comparison of spine and synaptic morphology revealed a lower spine density in control hemisphere ($F_{4,6}$ = 1.186, P<0.09ns) and in the stimulated hemisphere ($F_{4,6}$ = 1.204, P<0.03*) (Table: 6.1) in the stimulated hemisphere of the young rats, but a significant increase in synaptic density was observed in both the control ($F_{4,6}$ – 3.085, P<0.002*) and stimulated hemispheres ($F_{4,6}$ = 2.962, P<0.001*) of the young rats indicating that stimulation *per se* contributes to morphological changes (Table: 6.2).

Table 6.1: Mean Spine Density, Spine Length and Percentage of Branched Spines (± S.E) in the Middle Molecular Layer of Dentate Gyrus of Young and Aged Rats 45 Minutes after High Frequency Stimulation.

	Aged Rats		Young Rats
Spine Density	Decreased		Increased Significant
Spine Length	Longer		Shorter Significant
Branched Spines	Increased		Decreased Not Significant

Table 6.2: Mean Numerical Synaptic Density (of all synapse types), Spine Synapses, Shaft Syanpses and Percentage of Perforated Synapses in the Middle Molecular Layer of Young and Aged Rats 45 Minutes after High Frequency Stimulation. Vertical bars represent standard errors (± S.E).

	Aged Rats		Young Rats
Synaptic Density	Decreased		Increased Significant
Spine Synapses	Decreased		Increased Significant

Shaft Synapses	Increased		Decreased Not Significant
Perforated Synapses	Increased		Decreased Significant

Spine length was significantly higher in control ($F_{4,6}$ = 0.901, P<0.031*) and stimulated hemisphere ($F_{4,6}$ = 0.821, P<0.029*) of the aged rats (Fig: 6.1). The mean density of "spine" synapses was significantly higher in ($F_{4,6}$ = 2.61, P< 0.002*) and stimulated hemisphere ($F_{4,6}$ = 2.61, P<0.000001*) in the young rats (Fig: 6.2). However, the mean density of "shaft" synapses was higher in the stimulated hemisphere ($F_{4,6}$ =0.398, P<0.547ns) of aged rats, but the mean "shaft" synapse density was significant ($F_{4,6}$ = 0.514, P<0.04*) higher in the control hemisphere of young rats (Table: 6.2). An absolute increase in the percentage of branched spines (Table 6.1) and a significant increase in the percentage of perforated synapses (simple, complex and total) were observed in aged rats (Table: 6.2).

The present data complement the earlier findings by (deToledo, *et al.*, 1988, 1995) that the synaptic density is significantly lower in the aged rats compared to the young rats in both spatial learning and after LTP induction. The percentage of simple perforated synapses was consistently lower in the stimulated hemisphere. Consequently, simple perforated synapses could represent a structural intermediate between non-perforated and complex perforated synapses. The current results on the percentage of perforated synapses (simple, complex and total) differs from that of Geinisman, *et al.*, (1994), who observed a significant increase in multiple, completely partitioned transmission in young adult rats compared to the aged rats. This difference in the percentage of perforated synapses could be due the age of the young rats. A comparison of spine density, spine

length, percentage of branched spines and density of spine and shaft synapses was not possible, as precise data on these parameters was not available here. Based on the above findings it could be suggested that in aged rats the essential morphological parameters for sustaining LTP are significantly longer spines a higher density of "shaft" synapses, branched spines and perforated synapses (simple, complex, total). Aged rats with a lower number of these parameters are unable to exhibit the synaptic plasticity associated with sustaining LTP, as in young rats.

Possible mechanisms for spine and synaptic plasticity

One of the reasons for the study of anatomical plasticity within the hippocampus is the discrete lamination and segregation of cells and axons that permits the detection of changes in the pattern of connections. Furthermore, the connections and functions of the hippocampus are consistent with relatively simple models of neural networks that subserve learning and memory (McNaughton and Morris, 1987).

Morphological, alterations in the number of hippocampal neurons, spines and synapses have been widely investigated as structural substrates of memory disorders, and normal ageing (Moser, *et al.*, 1994, Barnes, *et al.*, 1980a; deToledo, *et al.*, 1984a). A number of hypothesis have been put forward to explain the mechanism of alteration in the efficacy of spines and synapses underlying electrically-induced LTP. Electrically-induced LTP has at least two components, one lasting for minutes to hours (acute), while the other lasting over days (chronic) in the hippocampal formation (Racine, *et al.*, 1983), thus suggesting more than one mechanism.

Electrophysiological investigations have not been able to access spines directly. Hence, biophysical models are able to shed some light into the functional analysis of spines. The

electrical resistance of the thin spine stalk could prolong the synaptic response generated by the spine head in the postsynaptic neuron Chang, *et al.*, (1952). The idea that values of the spine stalk resistance could change the efficacy of an axospinous synapse was introduced by Rall and Rinzel, *et al.*, (1971a,b).

Assuming the spine head to be a passive membrane (i.e., without voltage-sensitive ion channels), then the voltage at the parent dendrite (i.e. in the spine base) will be half of that at the spine head provided that the spine stalk resistance equals that of the dendritic branch resistance, on the other hand, if the spine head has an active membrane (i.e., with voltage-sensitive ion channels), an action potential generated at the spine head could result in amplification of the synaptic input at the parent dendrite (Perkel and Perkel, 1985). Therefore, the spine stalk has emerged as an important locus for alterations in synaptic efficacy, within an optimum range of resistance (Rall and Segel, 1988).

An active spine with synaptic response increases considerably the amplitude of the excitatory postsynaptic potential (EPSP) generated, subsequently increasing the amount of current that flows into the parent dendritic branch (Shepard, *et al.*, 1985). Stimulated spines spread currents that may activate sequentially neighbouring excitable spines by bringing them to their respective thresholds of activation, thus generating a saltatory propagation of an action potential in the dendritic branch (Shepard, *et al.*, 1985).

The spine-to-spine impulse communication and triggering of the response in the parent dendrite is critically dependent on morphological and physiological parameters of the spine stalk, such as its dimensions and electrical resistance (Koch and Poggio, 1983; Shepard, *et al.*, 1996; Shepard and Brayton, 1987; Ziv and Smith, 1996). Alterations in the stalk resistance could result from decreasing the stalk diameter, increasing the stalk

length, or partially occluding the stalk with membranes of the spine apparatus (SA) (Miller, *et al.*, 1985).

Why can certain aged rats sustain LTP, while others do not?

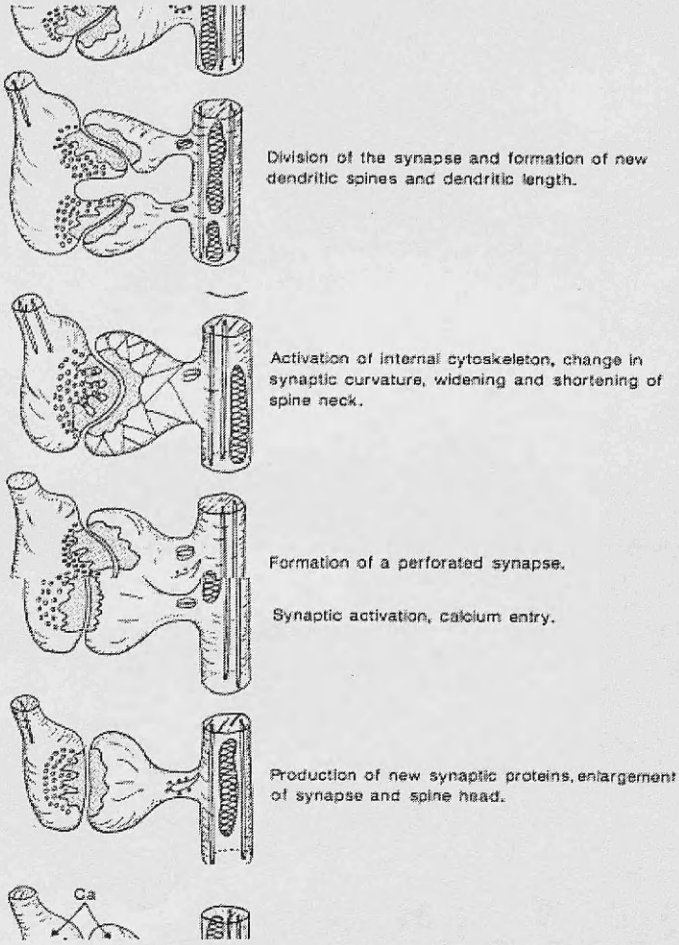
The experimental results between aged (potentiated and non-potentiated) indicates that the mean spine length was longer in the aged rats that sustained LTP. The principal biophysical factors that control the nature of the EPSP observed in (Fig 4.3) are the degree of cytoplasmic spine resistance to the resistance of the dendrite. Previous studies have shown that, the change to the parent dendrite is reduced by more than 10%, if the synaptic conductance was 5ns, since 33% of the spine stalks (in the CA1 region) are sufficiently thin (Harris and Stevens, 1989). Besides providing an electrical resistance to the current flow, the spine stalk also provides a diffusional resistance to the flow of ions and molecules. Synaptic activation of a spine, results in generation of a large electrochemical gradient that would attract charged molecules into the spine head from the dendrite (Horwitz, 1984; Yuste and Denk, 1995) and eventually controlling the diffusion of molecules in and out of the spine (Shepard, 1979; Shepherd, 1997). The spine stalk may effectively isolate the spine head, by restricting the flow of molecules, Ca^{2+} in particular and thus providing a localized environment in which reactions specific to a particular synapse would occur (Holmes, 1990). However, this large transient increase of Ca^{2+} attained in the spine head will be restricted to spines with long, thin stalks (Zador, *et al.*, 1990; Holmes, 1990). In conclusion, a key function of the spine stalk, in addition to the electrical resistance would be to faclitate increased EPSP and synaptic plasticity.

Finally what might be the mechanism of spine and synaptic remodelling (modification of preexisting spines or creation of new spines)?

The experimental data observed in young and aged rat's 45 minutes after LTP induction indicates that it is simpler for aged and probably young rats to modify the existing spines and synapses rather than constructs completely new ones by synaptogenesis. Since, it would take a longer time construct new spines. In the case of aged rats, the lower spine and synaptic densities suggests loss of spines and synapses due to ageing. Therefore, in order to sustain LTP after 45 minutes, modification of preexisting spines and synapses is a possible mechanism for synaptic efficacy, such a mechanism is termed as 'spine splitting'.

The Petit 'Spine Splitting' model takes into account the biophysical properties and calcium ion dynamics of dendritic spines. The entry of calcium, activates the internal cytoskeleton, production of new proteins, alterations in synaptic shape and size, the formation of perforated synapses by synaptic splitting, changes in the shape of the dendritic spine, the formation of new spines, and ultimately the addition of new dendritic spine (Fig 6.8).

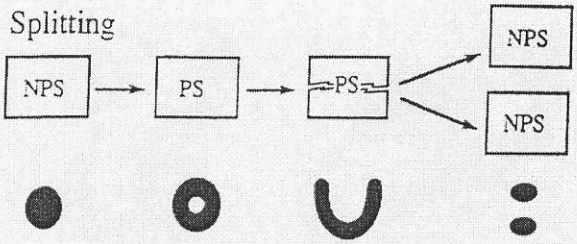
Figure 6.8: The 'Spine Splitting' model proposed by Petit, (1988).



Modifications in the structure of spines and synapses could of course be due the remodeling of pre-existing spines and axospinous junctions rather than building completely new ones. Perforated synapses and branched spines are known to augment synaptic efficacy, as twin spine heads never share the same presynaptic element. Therefore, the LTP induced increase in the percentage of branched spines and complex perforated synapses should have been accompanied by a corresponding loss of other synaptic subtypes that could be postulated to be structural intermediates in synaptic plasticity (Geinisman, *et al.*, 1994, 1995) (Fig 6.9). A decrease in spine-associated

polyribosomes was observed following the induction of LTP by Desmond and Levy, (1990). This suggests a possible remodelling of existing spines. This phenomenon is of special interest, because branched spines and perforated synapses have not only been associated with LTP induction (Trommald, *et al.*, 1990), but have also been regarded as potentially important indicators of age- dependent synaptic modification (Harris, *et al.*, 1992; de Toledo-Morrell, *et al.*, 1988; Petit, 1988).

Figure 6.9: The splitting hypothesis proposes that non-perforated synapses (NPS) are transformed into perforated synapses (PS), that subsequently for daughter non-perforated synapses (NPS) (after Jones, 1993)



In favour of the "spine splitting" hypothesis or "interconversion" hypothesis is the frequent inference in Golgi and EM studies that the extent of dendritic branching and the number of spines and synapses, are increased in trained animals (Spinelli, *et al.*, 1980) and following electrical stimulation (LTP) Rusakov, *et al.*, (1997); Sorra, *et al.*, (1998); Stewart, *et al.*, (1997); Dhanrajan, *et al.*, (1997, 1998).

Future Proposals

In the process of answering the question of the nature of anatomical plasticity observed in hippocampal dendritic spines and synapses after long-term potentiation (LTP) and ageing, this thesis has raised several further issues that are essential for understanding the mechanism of altered synaptic efficacy. It would be necessary to:

- Examine whether lateralisation of dendritic spines and synapses exists in both young and aged naïve rats using Golgi impregnation, serial EM sections and unbiased stereological techniques.
- Examine alterations in synaptic plasticity accompanying behavioural paradigms such as spatial learning (Morris Water Maze), or more physiological methods of potentiation such as theta stimulation in both young and aged rats using Golgi impregnation, serial EM sections and unbiased stereological techniques. The alterations in synaptic plasticity observed in these paradigms could further support LTP as a probable model for learning and memory.
- Undertake further studies at both earlier and later time points to clarify the issue of whether the described morphological change results from interconversion of existing synapses as suggested here, or from synaptogenesis at longer times post-LTP.
- Examine the expression of microtubule associated protein 2 (MAP-2), Ca^{2+}/calmodulin-dependent kinase II (CaMKIIα), activity regulated cytoskeletal protein (Arc), synaptophysin, synaptobrevin and synapsin in young and aged rats at 1hour, 6 hours, 12 hours and 24 hours after LTP induction using light, confocal and electron microscopy.

- Examine if dietary replenishment of arachidonic acid in aged rats would enhance the expression of MAP2, CaMKIIα, Arc, synaptophysin, synaptobrevin and synapsin at 1hour, 6hours, 12 hours and 24 hours after LTP induction using light, confocal and electron microscopy (Steward, *et al.*, 1998).
- Use Fluoresecent dyes such as FM1-43 and DiI to understand the dynamics of dendritic spines and synapses that are constantly being replaced during the course of ageing. These fluorescent dyes could indirectly distinguish recently altered synapses from those that were modified by past experience (Frey and Morris, 1997, 1998).
- Examine the role of cytoplasmic polyadenylation element (CPE) and cytoplasmic polyadenylation element binding protein (CPEB) in aged rats using light, confocal and electron microscopy. (Wu, *et al.*, 1998).

Appendix

Phosphate Buffer (PB)

Phosphate buffer was made from two stock solutions, Sodium dihydrogen orthophosphate [$NaH_2PO_4.H_2O$ - Mw 137.99] - solution A and Disodium hydrogen orthophosphate [Na_2HPO_4 - Mw 141.96] - solution B.

The solutions A and B are mixed in a 4:1 ratio to make up a stock solution of 0.2M phosphate buffer at pH 7.4.

Preparation of paraformaldehyde/glutaraldehyde fixative in phosphate buffer:

Materials:

- Paraformaldehyde (Agar Scientific Ltd, England)
- Glutaraldehyde, 25% vacuum distilled (Agar Scientific Ltd, England)
- 1M NaOH (Sigma,USA)
- 0.2M phosphate buffer

* Caution: The above fixative is prepared in a fume hood.

Distilled water (one-third of the final volume) is heated to 65 -70^{0}C. The required amount of paraformaldehyde powder is added to the distilled water and stirred with a magnetic stirrer for about 10-15 minutes. 1M NaOH is added gradually, until the solution becomes clear. An appropriate volume of glutaraldehyde is added to the solution and filtered, finally it is made up to half volume with distilled water. Before adding remaining half volume of 0.2M phosphate buffer to make up the final volume.

Perfusion

Materials:

- Anaesthetic (Sodium pentabarbitone, Urothene)
- Mixing chamber
- Rinsing solution (0.9% saline)
- Fixative (paraformaldehyde/glutaraldehyde)

Golgi Impregnation Technique:

Materials:

- 0.2M phosphate buffer
- Glucose (BDH, England)
- Osmium tertoxide (Agar Scientific)
- Calcium chloride ($CaCl_2$; Sigma, USA)
- Potassium dichromate ($K_2Cr_2O_7$; Sigma, USA)
- Silver nitrate ($AgNO_3$; Sigma, USA)
- Agar (Oxoid, England)
- graded series of alcohol [ethanol]
- xylene
- DPX mountant (BDH Chemicals Ltd; Poole, England)

Processing for Standard Transmission Electron Microscopy (TEM):

Preparation of ultrathin sections is essential, due to the limitations in the infiltration of electron beams through stained neuronal sections at accelerating voltages up to 80KV. The specimen should not be more than 100nm for optimum resolution. The whole procedure is to produce blocks with well preserved ultra structures at 80nm (Fig 7.2)

Materials:

- Fixative (2% paraformaldehyde/2% glutaraldehyde, Agar Scientific Ltd, England)
- 0.2M Phosphate Buffer
- Osmium tetroxide (Agar Scientific)
- Glucose
- Graded series of acetone
- Epon 812 (Agar Scientific Ltd, England)
- DDSA (Dodecenyl Succinic Anhydride, $C_{16}H_{25}O_3$, Agar Scientific Ltd, England)
- MNA (Methyl Nadic Anhydride, $C_{10}H_{10}O_3$, Agar Scientific Ltd, England)
- BDMA (N- Benzyldimethylamine, $C_6H_5.CH_2.N(CH_3)_2$, Agar Scientific Ltd, England)

Darkroom Techniques (Electron Microscopy):

The photographs were taken on JEOL 1010 electron microscope using AGFA Scientia EM film. The negatives were developed in AGFA B&W film developer (dilution 160ml of developer + 2500 ml of distilled water) at 20°C for 4 min, and fixed in Hypam Ilford 2000Rt fixer (dilution 1+4) for 3 min. Then the negatives were washed in water for 20 minutes and finally rinsed in the mixture of distilled water with few drops of wetting agent. The negatives were then left to dry in drying cabinet.

Photographs were enlarged with an Ilford Ilfospeed Multigrade 500S enlarger, printed on Ilford MGIV Multigrade IV RC deluxe photo print paper, and then processed on Ilford Ilfospeed 2240 print processor.

Equpiment Used:

Electron Microscope:	JEOL 1010
Ultramicrotomes:	Reichert OMUE and OMUE 4
Light Microscope:	Zeiss Axiophot, Nikon
Ultrastainer:	LKB Bromma 2168 ultrastainer Carlsberg system
Carbon Coater:	Edwards carbon coater
Photo enlarger:	Ilford Ilfospeed Multigrade 500S
Print processor:	Ilford Ilfospeed 2240
Tissue chopper:	Sorval tissue chopper

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